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Chaperone Functions of the E3 Ubiquitin Ligase CHIP*

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Meredith F. N. Rosser[‡1], Erin Washburn[‡], Paul J. Muchowski[§2], Cam Patterson[‡¶3], and Douglas M. Cyr[‡‖4]

From the [‡]Department of Cell and Developmental Biology, [¶]Carolina Cardiovascular Biology Center, and [‖]University of North Carolina Cystic Fibrosis Center, University of North Carolina Chapel Hill School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599 and the [§]Gladstone Institute of Neurological Disease, Department of Biochemistry and Biophysics and Department of Neurology, University of California, San Francisco, California 94158

The carboxyl terminus of the Hsc70-interacting protein (CHIP) is an Hsp70 co-chaperone as well as an E3 ubiquitin ligase that protects cells from proteotoxic stress. The abilities of CHIP to interact with Hsp70 and function as a ubiquitin ligase place CHIP at a pivotal position in the protein quality control system, where its entrance into Hsp70-substrate complexes partitions nonnative proteins toward degradation. However, the manner by which Hsp70 substrates are selected for ubiquitination by CHIP is not well understood. We discovered that CHIP possesses an intrinsic chaperone activity that enables it to selectively recognize and bind nonnative proteins. Interestingly, the chaperone function of CHIP is temperature-sensitive and is dramatically enhanced by heat stress. The ability of CHIP to recognize nonnative protein structure may aid in selection of slow folding or misfolded polypeptides for ubiquitination.

Cells must constantly monitor the folding status of nascent polypeptides and repair or degrade misfolded proteins during protein denaturing stress. To accomplish these tasks, the cell relies on an intricately regulated protein quality control (QC)[5] system. The cytosolic QC system consists of molecular chaperones, such as Hsp70 and Hsp40, that promote the proper folding and refolding of nonnative proteins and the ubiquitin proteasome system, which degrades misfolded or stress-damaged proteins (1–4). Maintenance of cellular homeostasis requires a delicate balance between that activity of protein folding and ubiquitin proteasome systems. Under circumstances where the molecular chaperone system is unable to promote proper folding of a protein substrate to its native state, it is necessary for the substrate protein to be selected for degradation, a process that is often referred to as protein triage (5, 6). Protein triage needs to be tightly regulated, since the escape of toxic proteins from QC systems or overactivity of protein degradation pathways leads to a variety of human diseases (7).

Partitioning of nonnative polypeptides between folding and degradation pathways appears to be influenced by the folding kinetics of individual proteins as well as by a network of co-chaperones that bind and regulate polypeptide binding and release by Hsp70 family members (8–13). CHIP is a co-chaperone that functions as an E3 ubiquitin ligase that links the polypeptide binding activity of Hsp70 to the ubiquitin proteasome system. CHIP binds Hsp70 through interactions between its N-terminal TPR domains and the C-terminal EEVD motif found on Hsp70. The binding of CHIP to Hsp70 can stall the folding of Hsp70 client proteins (14–16) and concomitantly facilitate the U-box dependent ubiquitination of Hsp70-bound substrates (16, 17). CHIP appears to play a central role in cell stress protection (18–20) and is responsible for the degradation of disease-related proteins that include cystic fibrosis transmembrane conductance regulator (15), p53 (21), huntingtin (22), ataxin-3 (22), Tau protein (23–25), and α-synuclein (26).

The domain structure of CHIP makes it uniquely suited to adapt the polypeptide binding activity of Hsp70 for use as the substrate selector of a multisubunit E3 ubiquitin ligase complex that contains Hsp40 and the E2, UbcH5 (16, 27). Although the ability of CHIP to utilize its U-box domain to attract UbcH5 to ubiquitinate Hsp70-bound substrates is established (11, 16, 17, 27), the mechanism by which different Hsp70 clients are selected for degradation by CHIP is not understood. This is an important question, because productive folding intermediates and misfolded proteins that are bound by Hsp70 appear to have similar conformations, and it does not appear that folding kinetics are the sole determinant of a nonnative protein's fate (5, 15, 28). If CHIP were able to selectively recognize nonnative protein structure, this would help explain how Hsp70 clients are selected for degradation.

Evidence to suggest that CHIP possesses chaperone activity comes from experimental data in which overexpression of CHIP or CHIPΔU-box in cultured cells was found to enhance the refolding of stress-damaged proteins (18) and activate the stress-dependent transcription factor, HSF, independent of stress (19). In addition, a number of Hsp70 co-chaperones possess an intrinsic polypeptide binding activity and act independently to suppress protein aggregation (29–31). Based on these observations, we tested whether CHIP could function as an autonomous molecular chaperone. We report that CHIP exhibits intrinsic chaperone function that enables it to bind nonnative substrates and suppress protein aggregation. In addi-

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[4] Work in this author's laboratory is supported by NIH Grant GM 056981 and the Cystic Fibrosis Foundation. To whom correspondence should be addressed: Dept. of Cell and Developmental Biology, School of Medicine, University of North Carolina, Chapel Hill, NC 27599. Tel.: 919-843-4805; E-mail: DMCYR@med.unc.edu.
[5] The abbreviations used are: QC, quality control; E3, ubiquitin-protein isopeptide ligase; PBS, phosphate-buffered saline; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; GdmHCl, guanidinium HCl; E2, ubiquitin carrier protein; GST, glutathione *S*-transferase.

tion, CHIP chaperone activity was found to be enhanced by heat stress. These data suggest that the ability of CHIP to function as a chaperone is an important feature of its action in protein triage and cell stress protection.

MATERIALS AND METHODS

Cell Culture—AD-HEK293 cells from Stratagene were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin; Invitrogen) at 37 °C in an atmosphere of 5% CO_2. Cell transfections were performed using Effectene reagent (Qiagen) with the indicated amounts of plasmid DNA.

Cell Culture Luciferase Assay—AD-HEK293 cells were transiently transfected with 1 μg of the luciferase-encoding plasmid, pGL3 (Promega), and with 0.25 μg of pCDNA3.1myc-CHIP. Transfected cells were allowed to recover for 24 h and were then treated with 25 μg/ml cycloheximide for 30 min at 37 °C. Following the cycloheximide incubation, cells were either maintained at 37 °C or heat-stressed at 45 °C for 30 min. Recovery periods of 0, 1, or 3 h at 37 °C were allowed, and then cells were assayed for luciferase activity according to the manufacturer's protocol (Promega) or were harvested by citric saline and lysed in an ATP-regenerating buffer consisting of 50 mM Tris-HCl (pH 8.0), 0.5% Nonidet P-40, 120 mM NaCl, 5 mM Mg-ATP, 40 mM creatine phosphate, and 0.25 mg/ml creatine kinase for immunoprecipitation analysis. Immunoprecipitations were carried out by the subsequent addition of goat α-luciferase antibody (Millipore) or rabbit α-Hsp70 antibody (SPA-757, Stressgen) and Protein G beads (Roche Applied Science). Immunoprecipitated proteins were visualized by Western blot.

Glutaraldehyde Cross-linking of Cell Lysates—AD-HEK293 cells transfected with pCDNA3.1myc-CHIP (0.5 μg) were lysed 24 h post-transfection in 50 mM Tris-HCl (pH 8.0), 0.5% Nonidet P-40, and 120 mM NaCl. Cell lysates were incubated at 4, 37, 42, or 45 °C for 15 min, and then either water or glutaraldehyde (0.025% final concentration) was added to the cell lysates (34). The cross-linking reaction was incubated for 10 min at 30 °C, and then reactions were stopped by the addition of 4× SDS-PAGE sample buffer. Samples were run on 10% gels and blotted for the presence of CHIP with an α-Myc antibody (Sigma).

Protein Purification Procedures—The following plasmids were used for overexpression of the indicated proteins in the BL21 *E. coli* strain: pET9d-Hdj2, pET11a-Hsc70 (32), pET11a-His_6UbcH5a, pET30-His_6CHIPK30A, pET30-His_6CHIPH260A, pET30-His_6CHIPP269A, pET11a-CHIP (16), pGST-hChip198–303 (14), and pGex6-HD53Q (33). Hsp70, Hdj-2, and His_6-tagged proteins were purified as previously described (32, 34, 35). Untagged CHIP was expressed by inducing BL21 cells expressing pet11-CHIP with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside. Cleared lysates were obtained by sonication and centrifugation at 20,000 rpm for 10 min, and then CHIP was purified by High Q anion exchange and hydroxyapatite chromatography (Amersham Biosciences). All of the above proteins were dialyzed into buffer A (150 mM NaCl, 20 mM K-Hepes, pH 7.4). Glutathione *S*-transferase (GST)-HD53Q-Myc proteins were purified from bacteria induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside by incubating the cleared lysate with GSH beads (Sigma) for 1 h at 4 °C, which were then washed three times with phosphate-buffered saline (PBS) (50 mM sodium phosphate, 150 mM NaCl, pH 7.4). Bound protein was eluted with 10 mM GSH, and samples were dialyzed in buffer containing 50 mM Tris-HCl (pH 7.0) and 150 mM NaCl.

Huntingtin Aggregation Assay—GST-HD53Q-Myc fusion protein (3 μM) was incubated at 30 °C with 2.5 units of PreScission protease (GE Healthcare) in Buffer B (50 mM Tris·HCl (pH 7.0), 150 mM NaCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 2 mM ATP, 50 mM KCl, 5 mM $MgCl_2$, 50 μg/ml creatine kinase, and 8 mM creatine phosphate) plus the indicated proteins for 3 h. Cleavage of the GST tag allows for aggregation and fibril formation to occur (33). Aggregation reactions were either stopped by the addition of an equal volume of a 4% SDS, 50 mM DTT solution followed by heating at 95 °C for 5 min for filter trap analysis or mixed with 4× native gel sample buffer (0.24 M Tris-HCl (pH 6.8), 40% glycerol, 1% Bromphenol Blue) and run on a 7% native gel. Filter trap analysis was carried out with cellulose acetate filters (0.2-μm pore size) using a Bio-Rad Slot Blot apparatus. HD-53Q-Myc protein was visualized by performing a Western blot using an α-Myc antibody (Sigma).

Luciferase Native Gel Assay—To examine the state of denatured luciferase, the luciferase was incubated in denaturation buffer (25 mM K-Hepes (pH 7.4), 50 mM KCl, 5 mM $MgCl_2$, 6 M guanidinium HCl (GdmHCl), 5 mM DTT) for 1 h at 25 °C. The denatured luciferase was then diluted into refolding buffer (25 mM K-Hepes (pH 7.4), 50 mM KCl, 5 mM $MgCl_2$, 1 mM ATP) with the indicated mixture of proteins and incubated for 20 min at 25 °C before the addition of 4× native sample buffer (0.24 M Tris-HCl (pH 6.8), 40% glycerol, 1% bromphenol blue) and application to a 7% native polyacrylamide gel.

In Vitro Oligomerization Assay—Proteins were diluted in Buffer A (150 mM NaCl, 20 mM K-Hepes (pH 7.4)) to a final concentration of 0.3 mg/ml. Samples were then incubated at either 4 or 42 °C for 15 min. 4× native sample buffer (0.24 M Tris-HCl (pH 6.8), 40% glycerol, 1% bromphenol blue) was added to each sample, and they were then applied to 7% native polyacrylamide gels, which were prepared according to the Laemmli method without SDS. The running buffer consisted of 50 mM Tris and 384 mM glycine. Gels were run for 3.5 h at a constant current of 15 mA.

Luciferase Aggregation Assay—Luciferase was diluted to a final concentration of 100 nM in reactions containing the indicated proteins in 120 mM NaCl, 40 mM K-Hepes (pH 7.4), 50 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, and 1 mM DTT. Aliquots of each reaction were stored on ice to represent total protein levels. Samples were incubated at room temperature for 5 min, followed by a 15-min incubation at 4 °C for native luciferase and a 15-min incubation at 42 °C for heat-denatured luciferase. Samples were centrifuged at 20,000 rpm (Beckman Allegra 64R centrifuge; 4 °C), after which the supernatants were separated from the pellet. Pellets were resuspended in a volume of 4% SDS, 350 mM β-mercaptoethanol, representing 5 times the volume that was originally centrifuged. The total aliquots and supernatants were also diluted 1:5 in 4% SDS, 350 mM β-mer-

captoethanol. All fractions were heated at 55 °C for 10 min and then were applied to a nitrocellulose membrane using a Bio-Rad slot blot apparatus. Western blots were performed using an α-luciferase antibody (Cortex).

Luciferase Holding and Refolding Assay—Refolding of chemically denatured firefly luciferase (Promega) was carried out as described previously (35). Briefly, luciferase (14.2 mg/ml) was diluted 42-fold into denaturation buffer (25 mM K-Hepes (pH 7.4), 50 mM KCl, 5 mM $MgCl_2$, 6 M guanidinium HCl, and 5 mM dithiothreitol). The denaturation reaction was allowed to proceed for 1 h at 25 °C, and then a 1-μl aliquot was removed and mixed with 125 μl of refolding buffer (25 mM K-Hepes (pH 7.4), 50 mM KCl, 5 mM $MgCl_2$, 1 mM ATP) that was supplemented with the indicated chaperone proteins and incubated at 25 °C. Where indicated, after 90 min, the reaction was split into two, and 1.5 μM Ssa1 and 5.5 μM Ydj1 was added to one half of the reaction, whereas Buffer A was added to the other half. The reactions were then incubated for another 30 min at 25 °C. Aliquots of 1 μl were removed from the folding reactions and mixed with 60 μl of luciferase assay reagent (Promega). Luciferase activity was then measured with a Turner TD-20/20 Luminometer.

Enzyme-linked Immunosorbent Assay (ELISA) to Measure Polypeptide Binding by Hsp70—A previously established ELISA was used to measure complex formation between Hsp70 proteins and denatured luciferase (36). Hsp70 was diluted into PBS. Then 100-μl aliquots of 50 nM Hsp70 solutions were added to the wells of microtiter plates. The Hsp70 was allowed to adhere to the walls of wells during a 1-h incubation at 25 °C. Wells were washed to remove unbound Hsp70 with 50 mM phosphate, pH 7.4, 150 mM NaCl, 0.02% Triton X-100 (PBST). Wells were blocked via a 1-h incubation with 200 μl of 1% bovine serum albumin in PBS. GdmHCl-denatured luciferase (0.4 μg) was diluted into reactions containing the indicated amounts of CHIP in PBS with 0.02% Triton X-100, 0.2% bovine serum albumin, and 1 mM ATP and then added to each well. After a 1-h incubation at 25 °C, the wells were washed three times with PBST. Luciferase retained in the wells was detected with a 1:5000 dilution of rabbit α-luciferase (Cortex Biochem, San Leandro, CA) in PBST. α-Luciferase was incubated in the wells for 1 h at 25 °C, and then following three washes with PBST, goat α-rabbit serum coupled with horseradish peroxidase was used to detect the luciferase antibody retained in the wells. Three more washes with PBST were performed to wash away any unbound secondary antibody. Hydrogen peroxide (0.05% final concentration) was added to a solution containing 0.22 mg/ml 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) in 50 mM sodium citrate, pH 4.0. This 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) solution was added to the wells of the microtiter plate, and color formation was measured at 405 nm with a microplate reader (Bio-Rad).

Rhodanese Aggregation Assay—Rhodanese aggregation was determined using light scattering as previously described (37). In brief, bovine rhodanese (50 μM; Sigma) was denatured for 1 h at 25 °C in 6 M guanidine HCl buffered with 10 mM Tris-HCl, pH 7.4, and 10 mM DTT. Upon denaturation, rhodanese was diluted 100-fold into 400 μl of reaction buffer containing 10 mM Tris-HCl, pH 7.4, 25 mM KCl, and 10 mM DTT. If included, chaperones were added to the reaction mixture prior to the addition of denatured rhodanese. In order to prepare the heat-shocked CHIP, an aliquot of CHIP was placed at 42 °C for 15 min and set on ice for 3 min before being diluted 10-fold into the reaction mixture. The rate of rhodanese aggregation was determined by observing the increase in light scattering in the sample over time using a spectrophotometer set at 320 nm.

Ubiquitination Assays —Ubiquitination assays were performed in a reaction buffer composed of 20 mM K-Hepes (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$, 4 mM ATP, 2 mM DTT, 0.5 mg/ml bovine ubiquitin (Sigma), 0.01 mg/ml rabbit E1 (Calbiochem), 8 μM UbcH5a, and 4 μM CHIP unless otherwise indicated. Additional proteins were added at the indicated concentrations. Incubations were performed at 37 °C for 3 h and terminated by the addition of 4× SDS sample buffer. Proteins were resolved on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and detected by blotting with the indicated antibodies.

RESULTS

Characterization of CHIP Activity during Heat Stress and Recovery—To expand our knowledge of CHIP action in protein triage, we sought to understand the enigmatic observation that overexpression of CHIP in cultured cells enhances refolding of heat-denatured luciferase (18). This result was puzzling, because purified CHIP functions with Hsp70 to polyubiquitinate misfolded luciferase (38) and therefore CHIP would be expected to clear denatured luciferase from the cell instead of enhancing its reactivation.

To determine the mechanism by which CHIP influences denatured luciferase activity, HEK-AD 293 cells were transfected with luciferase and CHIP expression plasmids. Then luciferase activity as well as complex formation between CHIP and luciferase were analyzed following heat denaturation. Heat stress reduced luciferase activity by almost 80% in control cells, and luciferase activity was partially recovered during a post-stress recovery incubation at 37 °C. Consistent with an earlier report (18), luciferase activity was consistently higher in cells overexpressing CHIP (Fig. 1*A*). However, the effect of CHIP on luciferase activity following heat stress was somewhat variable, because when identical experiments were carried out with high passage number cells (*i.e.* passage 50 *versus* passage 15), luciferase activity immediately following heat shock was decreased in response to CHIP overexpression (data not shown). However, in all cases, the rate at which luciferase activity was restored after heat stress was enhanced by CHIP overexpression. Western blots indicated that differences in luciferase activity observed were not due to the presence of different levels of total luciferase protein in different cell extracts (Fig. 1*B*). Thus, the presence of abnormally high levels of CHIP, which are likely to exceed the levels of its cognate E2 ubiquitin-conjugating enzyme, UbcH5, helps maintain a pool of denatured luciferase in a folding-competent and reactivable state.

Hsp70 and CHIP were both shown by co-immunoprecipitation to bind heat-stressed luciferase (Fig. 1*B*). Interestingly, the number of Hsp70-luciferase complexes that were isolated increased a few-fold in the presence of excess CHIP (Fig. 1*B*). The association of CHIP with luciferase appeared to be confor-

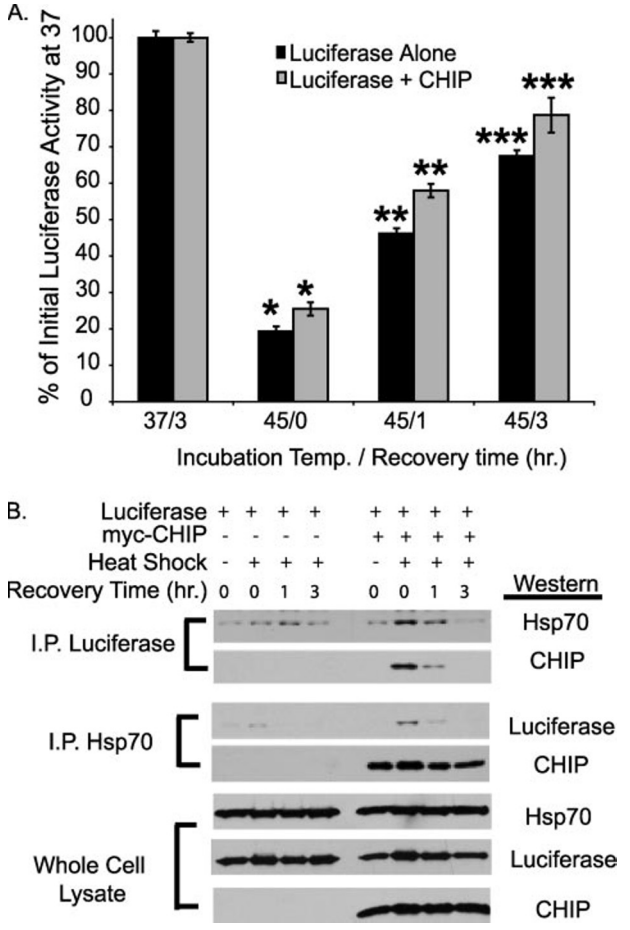

FIGURE 1. *A*, overexpression of CHIP protects luciferase activity after heat stress. *A*, AD-HEK 293 cells (passage 15) were transfected with 1 μg of the firefly luciferase-encoding plasmid, pGL3, with and without Myc-CHIP (0.25 μg). The cells were treated with cycloheximide (25 μg/ml) 24 h after transfection for 30 min at 37 °C to inhibit synthesis of new luciferase protein. The cells were then maintained at 37 °C or switched to 45 °C for 30 min and were harvested after a 0-, 1-, or 3-h recovery period at 37 °C. Cells were lysed in 1× reporter lysis buffer (Promega) and assayed for luciferase activity following the protocol provided by Promega. Results represent the average from three independent experiments and are plotted relative to the control activity without heat treatment. *p* values were calculated by Microsoft Excel using a one-way analysis of variance analysis comparing those cells that were and were not overexpressing CHIP (*, $p = 0.036$; **, $p = 0.002$; ***, $p = 0.019$). *B*, overexpression of CHIP does not result in an increased degradation of heat-denatured luciferase, but CHIP does specifically interact with heat-denatured luciferase and not refolded luciferase. Cells were transfected with the indicated plasmids at the same concentrations as used in *A* and then lysed in 4× sample buffer for whole cell lysates or in an ATP-regenerating buffer for immunoprecipitations (*I.P.*) with either luciferase antibody (Millipore) or Hsp70 antibody (Stressgen). Products of the immunoprecipitations were run on a 10% SDS-polyacrylamide gel and analyzed by Western blot with Myc, Hsp70, or luciferase antibodies.

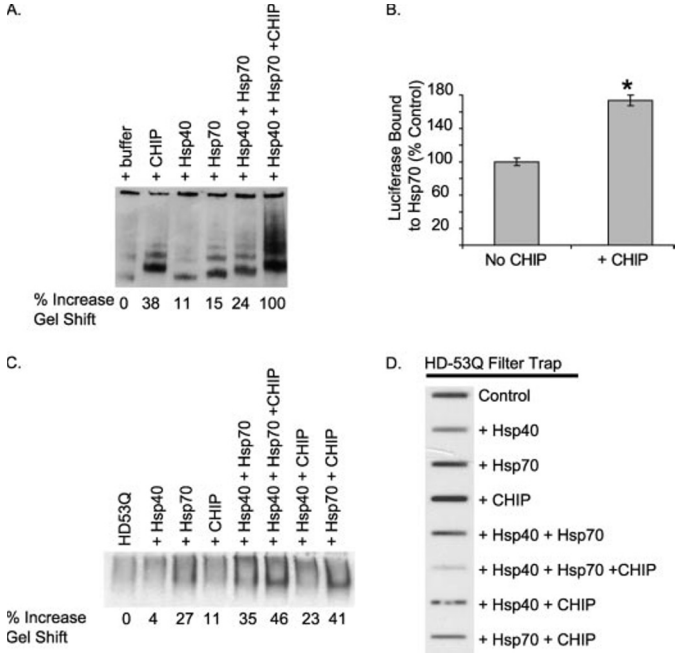

FIGURE 2. **CHIP enhances interactions between Hsp70 and substrate.** In *A*, luciferase was denatured in 6 M GdmHCl and then diluted into reactions containing the indicated proteins (0.615 μM Hsp40, 0.615 μM Hsp70, 2.5 μM CHIP). After a 20-min incubation at 25 °C, samples were mixed with 4× native sample buffer and loaded on a 7% native gel. Proteins were then transferred to nitrocellulose and probed using an α-luciferase antibody. A representative blot is shown, but quantitation indicated *below* represents the average from three independent experiments. *B*, ELISA to quantitate luciferase binding to Hsp70. The wells of a microtiter plate were coated with 5 pmol of Hsp70, washed, and blocked as described under "Materials and Methods." Luciferase was denatured in 6 M GdmHCl and then diluted into reactions containing no CHIP or 10 pmol of CHIP. These mixtures were then added to the microtiter wells, and the amount of luciferase that bound Hsp70 was determined as described under "Materials and Methods." Values represented are the average of three independent samples, and a *p* value of 0.0008 (*) was calculated for the difference between samples with and without CHIP using a one-way analysis of variance calculation in Microsoft Excel. *C* and *D*, GST-HD-53Q-Myc was incubated in cleavage buffer containing Precision Protease in the presence of the indicated proteins for 3 h at 30 °C (1.5 μM Hsp40, 3 μM Hsp70, 6 μM CHIP). The cleavage of the GST tag allowed for aggregation and fibril formation of the HD-53Q-Myc. The sample was split into two, and in *C* was mixed with 4× native gel sample buffer and run on a 7% native gel. Proteins were then transferred to nitrocellulose and visualized by Western blot with an α-Myc antibody. *D*, the other half of the sample from *C* was analyzed by filtering through a 0.2-μm pore size cellulose acetate membrane, and then protein aggregates that were large enough to be retained by the cellulose acetate were visualized by performing a Western blot with α-Myc antibody. Quantitations were performed using the Quantity One software from Bio-Rad.

mationally specific, because it was found to associate with inactive luciferase in heat-treated cells but not in controls where it was fully active (Fig. 1*B*). Indeed, as the luciferase refolded during the recovery period, the amount of CHIP isolated in a complex with luciferase decreased in a manner that was proportional to increases in luciferase activity.

Hsp70-dependent and -independent Effects of CHIP on Denatured Substrates—Whether the interactions between luciferase and CHIP are due to a direct binding of luciferase to CHIP or an effect of CHIP on Hsp70 binding to luciferase is difficult to evaluate in cultured cells. Therefore, we set out to determine if purified CHIP could function as a chaperone and bind nonnative polypeptides independent of Hsp70. Luciferase was chemically denatured with GdmHCl and then diluted into a refolding buffer containing CHIP, Hsp40, Hsp70, or different combinations thereof. Native gels were utilized to detect the amount of luciferase that formed a complex with the different chaperone proteins and therefore could enter the native gel matrix (Fig. 2*A*). In the absence of chaperones, negligible quantities of denatured luciferase entered the native gel matrix (Fig. 2*A*), which makes it difficult to quantitate the absolute percentage of luciferase that is bound by chaperone proteins. However, in comparison with the negative control (luciferase diluted into buffer), CHIP was able to bind and shift 38% (average value from three independent experiments) more luciferase into the gel, which is impressive when considering that the combination

of Hsp40 and Hsp70 together led to a 25% increase in luciferase migration into the gel (average value from three independent experiments). These results indicate that CHIP is able to bind a denatured substrate independent of interactions with Hsp70.

However, the most dramatic gel shift results were observed in the presence of Hsp40, Hsp70, and CHIP together. The combination of all three chaperones resulted in a 100% increase in the amount of luciferase migrating into the native gel matrix (average value from three independent experiments). This value surpasses that of a simple additive effect from the three proteins and suggests that Hsp40 and CHIP act jointly to enhance complex formation between Hsp70 and denatured luciferase. The apparent cooperative action of Hsp40 and CHIP in enhancing substrate binding by Hsp70 is consistent with the observation that the joint presence of purified CHIP, Hsp40, and Hsp70 is required for efficient E2-dependent polyubiquitination of luciferase (38).

In order to obtain more of a quantitative view of the effect of CHIP on Hsp70 substrate complexes, we utilized a modified ELISA assay to monitor luciferase binding to Hsp70 (36). Hsp70 was bound to the wells of a 96-well plate, and luciferase was added in the presence or absence of CHIP. The presence of CHIP resulted in a 73% increase in the amount of luciferase, which was found in complex with Hsp70 (Fig. 2*B*), which further supports the interpretation that CHIP can enhance the formation of Hsp70-substrate complexes. Together, the native gel shift results along with the ELISA results are consistent with the notion that CHIP is a chaperone and suggest that it can function via two mechanisms to modulate the fate of denatured luciferase. 1) CHIP can directly bind denatured substrates, and 2) CHIP can act in concert with Hsp70 to maintain denatured substrates in a soluble state.

To ascertain whether the ability of CHIP to act in concert with Hsp70 to maintain substrates in a soluble state is specific to luciferase or potentially a general mechanism of action, we examined this effect with another Hsp70 substrate, HD-53Q, which represents exon 1 of Huntingtin protein with an expanded polyglutamine region (33). Previously, Hsp70 was shown to suppress HD-53Q aggregation (33), and overexpression of CHIP has been shown to protect cells from polyglutamine aggregation in a U-box-independent fashion (39). The ability of CHIP and Hsp70 to maintain HD-53Q in a soluble state was determined with native gels and cellulose acetate filter trap assays (33). Native gel analysis demonstrates that HD aggregates do not readily enter the gel matrix, whereas soluble HD-53Q migrates into gels (Fig. 2*C*, *lane 1*). The inability of HD-53Q aggregates to enter native gels makes it difficult to quantitate the total extent to which chaperones suppress HD-53Q aggregation. Nevertheless, we can use this method to compare the relative ability of different chaperones to suppress HD-53Q aggregation and preserve the soluble state. The presence of Hsp70 maintains a portion of HD-53Q in a soluble state (*lanes 3* and *5*), but the addition of CHIP in combination with either Hsp40/Hsp70 or Hsp70 alone results in a much greater quantity of the HD-53Q protein entering the native gel matrix (Fig. 2*C*, *lanes 6* and *8*). These same samples were also analyzed by a cellulose acetate filter trap assay in order to detect large protein aggregates (33). The filter trap assay shows that although Hsp40 and Hsp70 are able to inhibit aggregate formation, this effect is greatly enhanced by the presence of CHIP (Fig. 2*D*, *lanes 5* and *6*). In these experiments, Hsp70 was present at levels where it was ineffective at independently suppressing the formation of large HD53Q aggregates. Thus, it is clear that CHIP can cooperate with Hsp70 to suppress HD-53Q aggregation. However, CHIP did not appear to independently bind HD-53Q and inhibit its aggregation (Fig. 2, *C* (*lane 4*) and *D* (*lane 4*)). The combination of the native gel and filter trap assays allows for visualization of soluble protein and of protein aggregates larger than 0.2 μm, but it must be noted that there may be smaller aggregates formed that are not detected by either assay. Regardless, the data in Fig. 2 show that CHIP is able to independently maintain denatured luciferase in a soluble state (Fig. 2*A*, *lanes 2* and *6*). However, CHIP appears to influence the dynamics of HD-53Q aggregation by enhancing the ability of Hsp70 to suppress the formation of large aggregates.

Luciferase is a typical globular protein that contains a number of hydrophobic motifs that are recognized by chaperones, which helps explain why CHIP can readily suppress its aggregation (40–42). HD-53Q is an atypical protein that is encoded by a version of exon 1 from the Huntingtin protein that is composed primarily of Gln residues and therefore may not be a high affinity substrate for CHIP (43). CHIP being inefficient at suppressing HD53Q aggregation yet still enhancing Hsp70 action in suppressing HD 53Q aggregation is consistent with the notion stated above that binding of CHIP to Hsp70 enhances Hsp70-polypeptide formation.

Suppression of Luciferase Aggregation by Purified CHIP—The native gel shift analysis demonstrated that CHIP can function as a chaperone to bind chemically denatured luciferase independent of Hsp70. Since CHIP was shown to protect luciferase from thermal denaturation in the context of the cellular milieu, the ability of CHIP to suppress the aggregation of thermally denatured luciferase was tested (Fig. 3). Heating luciferase to 42 °C causes it to denature and form an insoluble aggregate that will pellet upon high speed centrifugation (44). When CHIP was present during the denaturation step, it acted in a concentration-dependent manner to maintain heat-denatured luciferase in a soluble state and prevented the formation of pelletable luciferase aggregates (Fig. 3). These data further support the interpretation that CHIP possesses intrinsic chaperone activity and raise the question as to whether or not this chaperone activity is responsible for U-box-independent functions of CHIP (19, 18, 39).

To explore the subdomains of CHIP that are responsible for its chaperone function, the ability of purified point mutants in both its TPR (CHIP K30A) and U-box (CHIP H260A) domains was determined. Mutation of the TPR motif abrogated the *in vitro* chaperone activity, whereas the CHIP U-box mutant was still fully active (Fig. 3). In an extension of these studies, CHIPΔTPR (CHIP-(198–303)) was found to be unable to suppress luciferase aggregation, and surprisingly CHIPΔU-box (CHIP-(1–197)) was also inactive. Both the ΔTPR and the ΔU-box proteins have previously been shown to fold correctly such that CHIPΔTPR can function to bind E2 enzymes (45) and CHIPΔU-box can bind chaperones (6). However, neither of these CHIP truncation proteins were active in aggregation

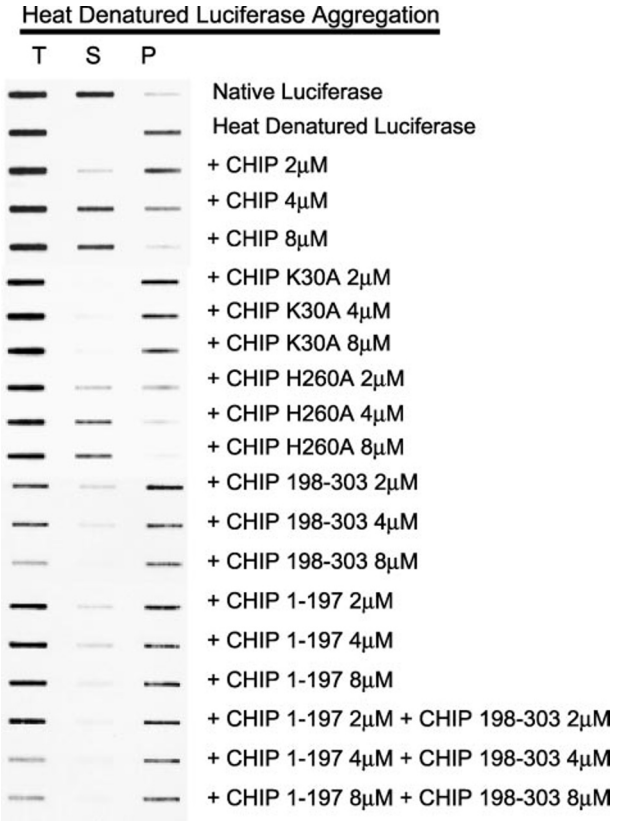

FIGURE 3. **CHIP acts as a chaperone to prevent aggregation of thermally denatured luciferase.** Luciferase was added to solutions containing the indicated proteins, and solutions were then incubated at room temperature for 5 min. During this incubation period, aliquots were taken to represent the total amount of luciferase present in each mixture. Native luciferase samples were then incubated on ice, whereas other samples were heat-denatured at 42 °C for 15 min. All mixtures were centrifuged at 20,000 rpm for 10 min in a Beckman Allegra 64R centrifuge, and the supernatants were separated from the pellets. Total (*T*), supernatant (*S*), and pellet (*P*) fractions were diluted in 4% SDS with 350 mM β-mercaptoethanol and applied to nitrocellulose using a Bio-Rad slot blot apparatus. Luciferase was visualized by blotting with α-luciferase antibody (Cortex).

assays when present individually or in combination (Fig. 3). The study of CHIP point mutants suggests that the polypeptide binding activity resides in its TPR domain. However, fragments of CHIP that lack either of these domains are not functional as chaperones. Since the overexpression of CHIP ΔU-box protects cultured cells from proteotoxicity (18), the inability of CHIPΔU-box to suppress protein aggregation was a surprise. However, since CHIPΔU-box can still bind Hsp70, it may exert its cytoprotective effects by modulating substrate binding to Hsp70. Overall, these data suggest that the TPR domains play an important role in the function of CHIP as a molecular chaperone.

Heat-dependent Enhancement of CHIP Chaperone Activity—Many chaperones exhibit increased chaperone activity upon exposure to heat stress (HS) (46–48). Therefore, upon demonstration of a chaperone activity for CHIP, the effect of heat treatment on CHIP was also determined. Chemically denatured luciferase was used in aggregation assays to directly compare the chaperone activity of CHIP incubated at 25 or 42 °C for 15 min (HS CHIP). We found that heat treatment greatly enhanced the ability of CHIP to function as a chaperone and prevent aggregation of the chemically denatured luciferase (Fig. 4*A*). We also noted a slight difference between the ability of HS CHIP to suppress aggregation of heat-denatured *versus* chemically denatured luciferase (compare Fig. 3 (*row 5*) and Fig. 4*A* (*row 3*)). The HS CHIP was more efficient at suppressing the aggregation of the heat-denatured substrate, potentially due to the fact that CHIP is present during the denaturation stage and may be able to trap intermediates before the luciferase completely unfolds.

Co-immunoprecipitation experiments demonstrate that when chemically denatured luciferase is diluted into buffer containing either CHIP or HS CHIP, a significant level of luciferase binding above background is observed for both conditions. However, heat treatment increases complex formation between CHIP and chemically denatured luciferase 3-fold (Fig. 4*B*).

Since heat treatment enhances the ability of CHIP to bind luciferase, we set out to determine if heat treatment would also enhance the ability of CHIP to inhibit polyglutamine aggregation. Previously, CHIP was observed to only inhibit HD-53Q aggregation in the presence of Hsp70 and not on its own (Fig. 2, *C* and *D*), yet heat treatment of CHIP enhanced its concentration-dependent ability to suppress aggregation of HD-53Q (Fig. 4*C*). Furthermore, HS CHIP functioned in a manner similar to the Hsp40, Ydj1, in the suppression of chemically denatured rhodanese (37) (Fig. 4*D*), whereas a similar concentration of CHIP was less active than HS CHIP in suppressing rhodanese aggregation. Thus, we have demonstrated with three different substrates that heat treatment enhances the ability of CHIP to suppress protein aggregation.

Effect of Heat Treatment on CHIP Oligomeric Status and Function—Heat stress-induced enhancement of the polypeptide binding activity of a specific chaperone has been correlated with changes in the oligomeric state of the chaperone protein (46–49). Therefore, the oligomeric state of CHIP in extracts from control (37 °C) and heat-treated (42 and 45 °C) cells was probed with the aid of glutaraldehyde cross-linking and SDS-PAGE (Fig. 5*A*). Cells expressing Myc-CHIP were lysed, the extracts were split in half, and 0.025% glutaraldehyde was added to one aliquot and water to the other. Upon analyzing both cross-linked and noncross-linked samples on SDS-polyacrylamide gels, it was observed that regardless of heat treatment, when the cell lysates were not cross-linked with glutaraldehyde, the CHIP migrated at the expected molecular mass of a CHIP monomer (36 kDa). In cell lysates that were not heat-shocked, the glutaraldehyde cross-linking resulted in a shift in the CHIP molecular weight to that of a dimer, as expected from previous reports (50, 51). However, after a heat shock of cells at 42 or 45 °C, CHIP was shown to form a high molecular weight complex, which upon cross-linking migrates at the top of the gel. In fact, after the 45 °C incubation, both the monomer and dimer forms of CHIP disappeared from the cross-linked sample, although the same decrease in total CHIP protein is not observed in the control. This suggests that the 45 °C heat shock causes CHIP to enter a large complex that cannot migrate into the gel. These large complexes observed after heat shock may represent a change in the homo-oligomeric status of CHIP, and this change in state may account for its increased ability to suppress protein aggregation. However, it is also possible that

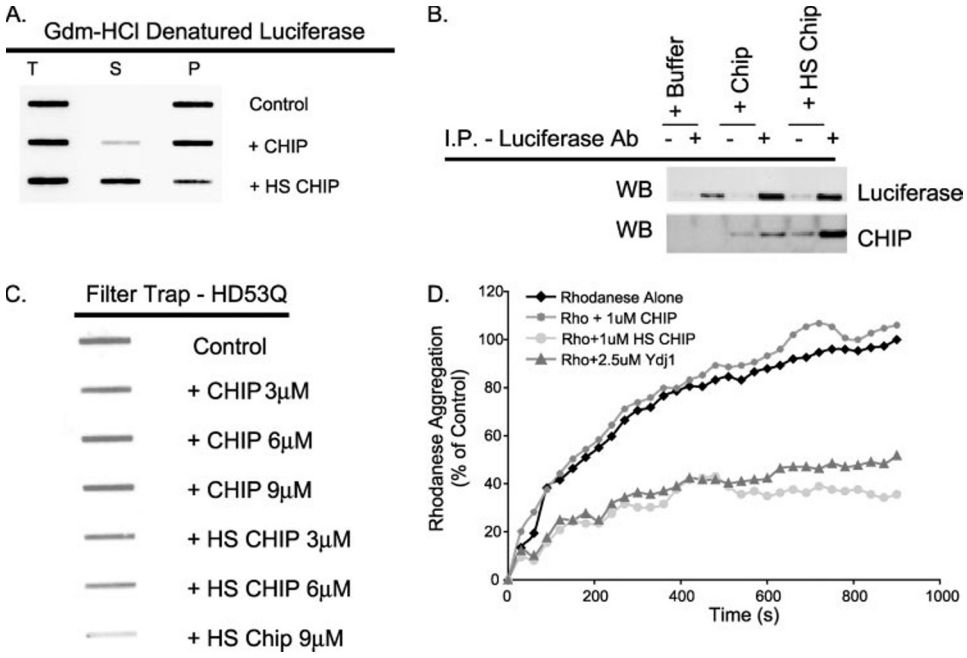

FIGURE 4. **Heat treatment enhances the chaperone activity of CHIP.** *A*, luciferase was chemically denatured using 6 M GdmHCl (dluc) and then diluted into reactions containing Buffer A alone or an 8 μM solution of proteins that were either preincubated at 4 °C (*CHIP*) or at 42 °C (*HS CHIP*) and then cooled on ice. Luciferase samples were then incubated at 25 °C for 20 min, fractionated into total (*T*), supernatant (*S*), and pellet (*P*) fractions, and visualized by Western blot with α-luciferase antibody (Cortex). *B*, heat treatment increases the affinity of CHIP for denatured luciferase. Reactions were prepared as in *A*, except after the 20-min incubation at 25 °C, 1 μl of luciferase antibody (*Ab*) was added to samples where indicated, and complexes were then isolated with Protein G-agarose beads. The samples lacking luciferase antibody serve as a negative control for background binding of proteins to Protein G-agarose beads. *C*, HD-53Q aggregation is inhibited by heat-treated CHIP. GST-HD53Q-Myc (1.5 μM) was incubated in cleavage buffer containing the indicated concentrations of CHIP protein, which was preincubated at either 4 °C (*CHIP*) or 42 °C (*HS CHIP*) for 15 min. Precision Protease was then added to the cleavage reactions, and they were incubated for 3 h at 30 °C and stopped by the addition of a 4% SDS, 50 mM DTT solution. Samples were applied to a 0.2-μm cellulose acetate filter using a Bio-Rad slot blot apparatus, and HD53Q aggregates were visualized by Western blot with α-Myc antibody. *D*, rhodanese was denatured in 6 M GdmHCl for 1 h and then diluted into buffer with and without chaperones. Aggregation kinetics were measured by light scattering at a wavelength of 320 nm. *I.P.*, immunoprecipitation; WB, Western blot.

heat shock may drive the entrance of CHIP into large multiprotein complexes that contain other cellular proteins and denatured proteins.

In order to directly determine whether CHIP undergoes heat-induced oligomerization, we analyzed the affect of heat stress on purified CHIP proteins by native gel electrophoresis (Fig. 5*B*). Heat treatment at 42 °C for 15 min caused both wild type CHIP and CHIP H260A, a U-box mutant, to form high molecular weight oligomers, whereas the CHIP TPR mutant, CHIPK30A, CHIPΔTPR(CHIP-(198–303)), and CHIPΔU-box (CHIP-(1–197)) proteins remained at the same molecular weight as observed in controls (Fig. 5*B*). Thus, forms of CHIP that do not act to suppress protein aggregation in a heat-dependent manner also fail to oligomerize after heat stress (Figs. 3 and 5*B*). The ability of CHIP to oligomerize correlates well with the temperature-sensitive enhancement of its chaperone activity.

Since heat treatment causes a conformational change in CHIP that results in oligomerization, we sought to verify that heat stress does not affect the global conformation of CHIP in such a way to disrupt its Hsp70 binding or ubiquitin ligase activity. The functionality of heat-treated CHIP was tested using polyubiquitination assays, since wild-type CHIP proteins promote the E2-dependent formation of polyubiquitin chains when mixed with ubiquitin, ATP, E1, and the E2, UbcH5a (17). Heat treatment does not diminish the ability of CHIP, CHIP K30A, or CHIP-(198–303) to stimulate UbcH5a to assemble polyubiquitin chains (Fig. 5*C*). CHIP P269A was expected to be inactive, because it has a defective U-box and does not stimulate the ability of UbcH5 to form polyubiquitin chains (52). HS CHIP is also able to polyubiquitinate Hsp70 in a manner similar to CHIP (Fig. 5*D*), indicating that HS CHIP is not denatured, since it retains its ubiquitination activity as well as its ability to interact with Hsp70. Overall, these data show that heat stress induces a conformational change in CHIP that enhances the intrinsic chaperone activity of the protein but does not affect other CHIP activities.

CHIP Maintains Substrates in a Ubiquitination-competent State—CHIP has the ability to maintain substrates in a soluble state both through an interaction with Hsp70 and through an intrinsic chaperone activity. Therefore, we set out to determine how this activity is related to its function in protein triage. To establish whether the ability of CHIP to maintain denatured substrates in a soluble fashion affects the ability of those substrates to be ubiquitinated, both "holding" and "folding" assays (35) were performed in parallel with ubiquitination assays (Fig. 6). CHIP and HS CHIP exhibited negligible ability to refold GdmHCl-denatured luciferase (Fig. 6A). However, CHIP functioned in a temperature-dependent manner to maintain or hold denatured luciferase in a folding-competent state (Fig. 6*B*). When luciferase was diluted into buffer alone and incubated for 90 min, it aggregated and therefore could not be refolded upon the addition of Hsp40 and Hsp70. Strikingly, when denatured luciferase was diluted into mixtures containing CHIP, which was preincubated at a variety of temperatures, CHIP maintained the luciferase in a foldable state over the 90-min primary incubation, because a significant portion of luciferase could be refolded upon the addition of Hsp40 and Hsp70. CHIP, which was preincubated at 37 °C had approximately half the holding activity of the Hsp40 folding factor, Ydj1 (35, 37), whereas the holding activity of CHIP preincubated at 42 °C matched that of Ydj1.

Since CHIP is able to maintain heat-denatured luciferase in a foldable form, by analogy we hypothesized that this chaperone activity should hold luciferase in a ubiquitination-competent conformation. To test this, the ability of luciferase to be ubiq-

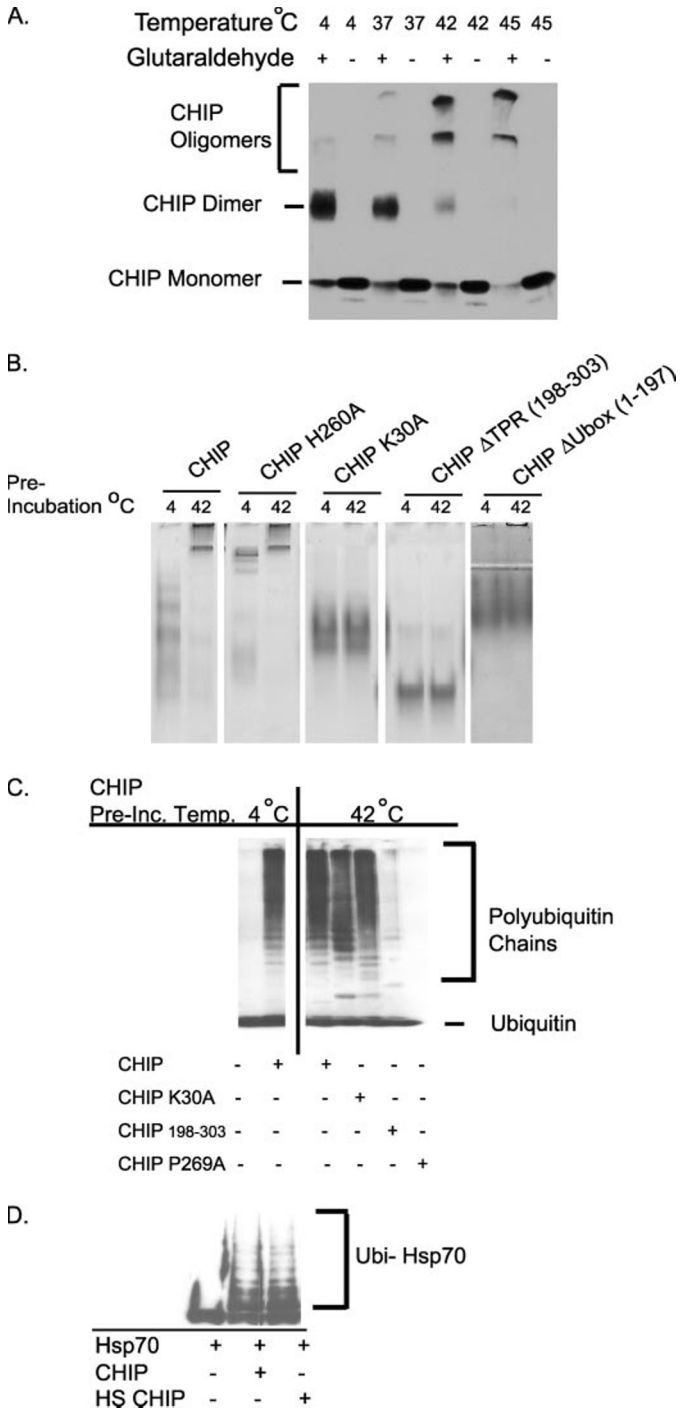

FIGURE 5. **Characterization of heat-treated CHIP.** *A*, AD HEK293 cells were transfected with pCDNA Myc-CHIP (0.5 μg) and 24 h post-transfection were lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 0.5% Nonidet P-40, and 120 mM NaCl. Lysates were then treated at either 4, 37, 42, or 45 °C for 15 min. After this incubation, glutaraldehyde was added to the cell lysates at a final concentration of 0.025%, and samples were incubated for 10 min at 30 °C. The cross-linking reaction was stopped by the addition of sample buffer, the samples were run on a 10% SDS-polyacrylamide gel, and CHIP was visualized by Western blot with an α-Myc antibody (Sigma). *B*, native polyacrylamide gels were used in order to determine the oligomeric status of CHIP proteins before and after heat treatment *in vitro*. CHIP was diluted in Buffer A to a concentration of 0.3 mg/ml and incubated at 4 or 42 °C for 15 min. Samples were then mixed with 4× native sample buffer and applied to a 7% polyacrylamide native gel, and proteins were visualized by Coomassie Brilliant Blue stain. *C*, CHIP proteins are still functional after heat treatment. CHIP proteins (8 μM) were preincubated at the indicated temperature for 15 min and then cooled on ice for 5 min. CHIP samples were then diluted 2-fold into ubiquitination reactions containing a final concentration of 20 mM K-Hepes (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$, 4 mM ATP, 2 mM DTT, 0.5 mg/ml bovine ubiquitin (Sigma), 0.01 mg/ml rabbit E1 (Calbiochem), and 8 μM UbcH5a. Ubiquitination reactions were incubated for 3 h at 37 °C before the addition of 4× sample buffer. Proteins were run on 10% gels and transferred to nitrocellulose for visualization by Western blot with an α-ubiquitin antibody. *D*, CHIP and HS CHIP both interact with and ubiquitinate Hsp70. CHIP (25 μM) was preincubated at either 4 or 42 °C and then diluted to a final concentration of 4 μM in ubiquitination reactions containing the above components plus 1 μM Hsp70 and 2 μM Hdj-2 (Hsp40). Ubiquitination reactions were incubated for 3 h at 37 °C before the addition of 4× sample buffer. Proteins were run on 10% gels and transferred to nitrocellulose for visualization by Western blot with an α-Hsp70 antibody (SPA-757; Stressgen).

uitinated after heat denaturation in the presence or absence of CHIP was investigated. After heat denaturation of luciferase ubiquitin, ATP, E1, and the E2, UbcH5a, were added to the luciferase reactions. In addition, non-heat-treated CHIP was included in samples that did not contain CHIP during the initial denaturation step. When CHIP was present during the initial heat denaturation step instead of added afterward, an increased level of luciferase ubiquitination was observed (Fig. 6*C*). HS does not appear to enhance the E3 activity of CHIP, because CHIP and HS CHIP stimulate the ability of UbcH5a to catalyze polyubiquitin chain assembly or ubiquitinate Hsp70 to the same degree (Fig. 5, *C* and *D*).

The ability of CHIP to suppress protein aggregation is sufficient to maintain luciferase in a soluble conformation that can be acted upon by chaperones or ubiquitin-conjugating enzymes. However, it is noteworthy to point out that the luciferase ubiquitination reactions shown were carried out in the absence of Hsp40 or Hsp70. Thus, in instances where CHIP can bind a nonnative substrate independent of Hsp70, it can also cooperate with UbcH5a to ubiquitinate those substrates independent of Hsp70. The extent to which CHIP functions independently of Hsp70 in cells to facilitate protein ubiquitination requires further study, but there may be instances where this occurs.

DISCUSSION

Data presented are the first to classify CHIP as an E3 ligase that functions autonomously as a temperature-sensitive molecular chaperone. CHIP was found to selectively bind nonnative proteins and maintain them in a soluble state that could subsequently be ubiquitinated by the E2 UbcH5. Thus, the ability of CHIP to recognize nonnative structure is an additional feature of its role in protein triage, and we envision scenarios in which CHIP would utilize its chaperone activity in both Hsp70-dependent and -independent processes. The integrated chaperone, co-chaperone, and ubiquitin ligase activities of CHIP may play a role in selection of slow folding or misfolded Hsp70 clients for ubiquitination and proteasomal degradation. CHIP also has the potential to utilize its intrinsic polypeptide binding activity to act independently of Hsp70 to select nonnative substrates for ubiquitination. This latter scenario helps to explain how direct binding of CHIP to Smad1 regulates the half-life of Smad1 (53, 54).

The mechanistic details of how interactions between CHIP and Hsp70 regulate the Hsp70 polypeptide binding and release cycle are not clear and are open to alternate interpretations (4,

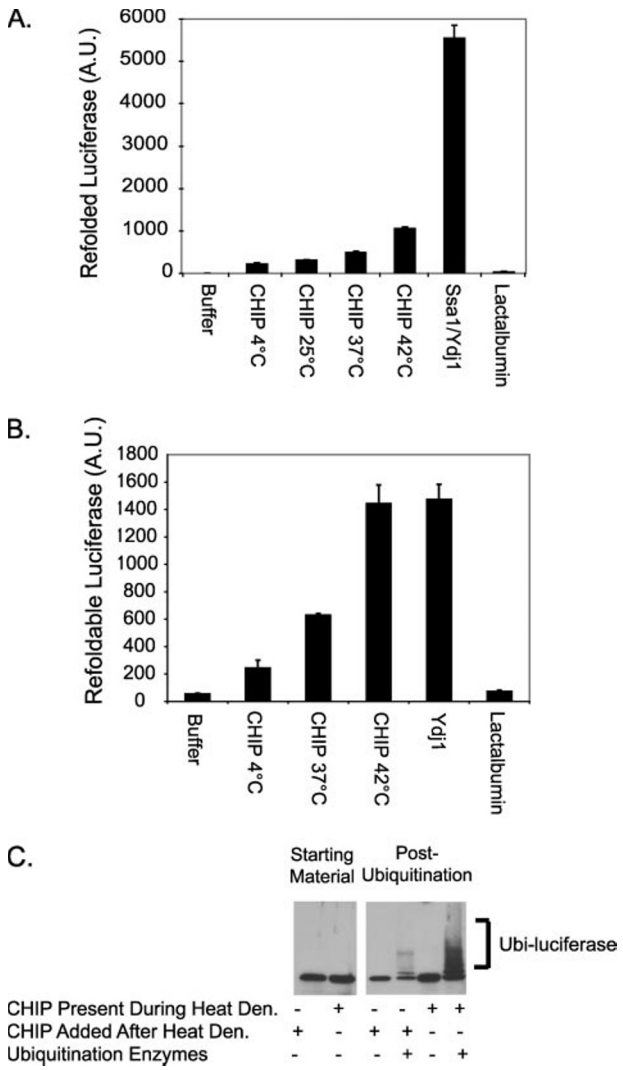

FIGURE 6. **CHIP maintains denatured luciferase in a folding- and ubiquitination-competent state.** *A*, CHIP does not refold denatured luciferase to a native state. Luciferase refolding assays were performed as described under "Materials and Methods." Briefly, luciferase was denatured in 6 M GdmHCl and then diluted into samples containing CHIP proteins that had been preincubated at the indicated temperatures. The folding reactions were then incubated for 90 min at 25 °C, and the luciferase activity was measured on a Turner luminometer. The *graph* represents the average values obtained from two independent experiments. *B*, luciferase was denatured in 6 M GdmHCl and then diluted into reactions containing the indicated proteins. After a 25 °C, 90-min incubation, the sample was split in half such that either buffer A or a mixture of Ssa1/Ydj1 (final concentrations of 1.5 and 5.5 μM, respectively) was added to each half. The buffer served as a negative control, whereas Ssa1 and Ydj1 are chaperones that will readily fold any luciferase that still remains in a folding-competent conformation. Samples were further incubated for 30 min, at which time luciferase activity was measured on a luminometer. The activity from the luciferase sample that received the buffer for refolding was considered background and was subtracted from the activity measured in the presence of Ssa1 and Ydj1. The *graph* represents the average of two independent experiments, and *error bars* represent the S.D. *C*, if CHIP is present during the heat denaturation of luciferase, it can maintain the luciferase in a ubiquitination-competent state. Luciferase was first diluted into reactions containing either buffer A or 2 μM CHIP, and aliquots were taken in order to show the amount of luciferase in the starting material. Luciferase reactions were then subjected to thermal denaturation by incubating at 42 °C for 15 min and then diluted into a secondary ubiquitin reaction mixture and incubated at 37 °C for 3 h (postubiquitination). All secondary reaction mixtures contained 20 mM K-Hepes (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$, 4 mM ATP, 2 mM DTT, 0.5 mg/ml bovine ubiquitin (Sigma), and, where indicated, the E1 (0.01 mg/ml rabbit E1 (Calbiochem)) and E2 (8 mM UbcH5a) ubiquitination enzymes. If CHIP was present during the initial heat denaturation step, then no additional CHIP was added to the secondary reaction mixture, but if CHIP was absent from the first reaction, then it was added to the secondary reaction at an equivalent final concentration (1 μM). Reactions were stopped by the addition of 4× SDS sample buffer, the samples were run on a 10% gel, and the proteins were transferred to nitrocellulose. Luciferase was visualized by Western blot with an α-luciferase antibody (Cortex).

18). We interpret our data to suggest that the coupled TPR-dependent binding of CHIP to Hsp70 and nonnative proteins serves to stabilize Hsp70-polypeptide complexes and thereby drive the assembly of the CHIP E3 ubiquitin ligase complex. The ability of CHIP to enhance Hsp70-polypeptide complex formation is supported by the following observations. 1) In the presence of overexpressed CHIP, an increase in the quantity of immunoprecipitable Hsp70-substrate complexes was detected (Fig. 1) (18), and 2) the addition of purified CHIP to mixtures that contained Hsp70 and denatured polypeptide led to an increase in Hsp70-polypeptide complex formation, as demonstrated by the native gel analysis and ELISA (Fig. 2). This CHIP action appeared to be due to an effect of CHIP on the stability of preformed Hsp70-substrate complexes, because native CHIP was not able to independently suppress HD-53Q aggregation but enhanced the ability of Hsp70 to suppress aggregation severalfold. Finally, the proposed action of CHIP in stabilizing Hsp70-polypeptide complexes is consistent with the ability of CHIP to reduce Hsp40-stimulated Hsp70 ATPase activity (14). The ADP form of Hsp70 has a high affinity for substrates, and if CHIP were to stabilize Hsp70-polypeptide complexes, this would slow the regeneration of the ATP form of Hsp70. A more detailed study of interaction between Hsp70, CHIP, and model substrates is required to prove the above interpretations of our data.

Since CHIP chaperone functions are enhanced by heat stress, CHIP may also have added roles during times of cellular stress. In fact, the enhanced ability of heat stressed CHIP to interact with denatured substrates may aid in its ability to protect cells and whole organisms from heat stress (18–20). In addition to interacting with Hsp70 to clear stress-damaged protein from cells, it is possible that CHIP utilizes its chaperone functions to interact with proteins such as HSF to regulate cell stress response (19, 20).

The mechanism by which heat enhances CHIP chaperone function is not clear. It is likely that the temperature-dependent oligomerization of CHIP and the formation of a multivalent chaperone account for its enhanced polypeptide binding activity. However, it is also possible that heat drives a conformational change that increases the exposure of its polypeptide binding site. Nevertheless, numerous other chaperones, such as the small HSPs, have been demonstrated to respond to their cellular environment with a change in oligomeric status and a corresponding change in chaperone activity (49, 55–59). The observations of CHIP change in oligomeric status and corresponding enhanced chaperone activity fit well with observations that indicate that CHIP is a dynamic protein that assumes more than one distinct conformation (45, 51). When mouse CHIP was co-crystallized with a C-terminal Hsp90 peptide that contained an EEVD motif, CHIP was found to exist as an asymmetric dimer (45). In contrast, the crystallization of a zebrafish CHIP construct that was missing the TPR domains but con-

tained the helical linker and U-box domains revealed a symmetric dimer (51). The differences in CHIP structure might be due to the fact that the zebrafish CHIP was crystallized in the absence of the TPR domains. However, it is also possible that the TPR domains of CHIP are not only required for binding to Hsp70 but may also serve to regulate or facilitate other aspects of CHIP activity.

Indeed, data presented demonstrate that CHIP TPR mutants do not exhibit the same degree of conformational plasticity as CHIP. A single point mutation in the TPR domain, K30A, or deletion of the TPR of CHIP prevents heat-induced oligomerization of CHIP and blocks its chaperone activity. These data suggest that either a particular conformation of the TPR domain is necessary for the protein to adopt the chaperone active state or that the TPR domain itself is where the polypeptide binding activity is localized. The CHIP polypeptide binding site remains to be identified, but the crystal structure of CHIP solved by Zhang *et al.* (45) has identified solvent exposed hydrophobic patches on the surface of the TPR domain that have the potential to interact with nonnative polypeptides (41).

In light of our data about the lack of chaperone activity of the CHIP TPR mutants, those who study CHIP need to be careful when interpreting experiments in which TPR mutants are utilized. This is the case because CHIP TPR mutants are unable to interact with Hsp70 and are used as a control to demonstrate that a specific activity is attributable to the interaction of CHIP with Hsp70. However, since CHIP is a chaperone and since its chaperone function is dependent upon the TPR domain, new controls will need to be carried out to assure that CHIP is acting through Hsp70 to modulate the activity of specific substrates.

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