Overexpression of the Cochaperone CHIP Enhances Hsp70-Dependent Folding Activity in Mammalian Cells

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CHIP is a cochaperone of Hsp70 that inhibits Hsp70-dependent refolding in vitro. However, the effect of altered expression of CHIP on the fate of unfolded proteins in mammalian cells has not been determined. Surprisingly, we found that overexpression of CHIP in fibroblasts increased the refolding of proteins after thermal denaturation. This effect was insensitive to geldanamycin, an Hsp90 inhibitor, and required the tetratricopeptide repeat motifs but not the U-box domain of CHIP. Inhibition of Hsp70 chaperone activity abolished the effects of CHIP on protein folding, indicating that the CHIP-mediated events were Hsp70 dependent. Hsp40 competitively inhibited the CHIP-dependent refolding, which is consistent with in vitro data indicating that these cofactors act on Hsp70 in the ATP-bound state and have opposing effects on Hsp70 ATPase activity. Consistent with these observations, CHIP overexpression did not alter protein folding in the setting of ATP depletion, when Hsp70 is in the ADP-bound state. Concomitant with its effects on refolding heat-denatured substrates, CHIP increased the fraction of nascent chains coimmunoprecipitating with Hsc70, but only when sufficient ATP was present to allow Hsp70 to cycle rapidly. Our data suggest that, consistent with in vitro studies, CHIP attenuates the Hsp70 cycle in living cells. The impact of this effect on the fate of unfolded proteins in cells, however, is different from what might be expected from the in vitro data. Rather than resulting in inhibited refolding, CHIP increases the folding capacity of Hsp70 in eukaryotic cells.

The 70-kDa heat shock protein Hsc70/Hsp70 is an abundant cytosolic and nuclear chaperone that assists many protein-folding events as well as facilitating the proteolytic degradation of unstable or misfolded proteins (5, 11, 21, 26, 38). These roles of Hsp70 rely on its ability to bind to short exposed hydrophobic stretches of substrate polypeptides in an ATP-regulated fashion. At normal growth temperatures, ATP-bound Hsp70 exhibits low affinity and fast exchange rates for substrates, whereas the ADP-bound state has high substrate affinity but slow exchange rates (9, 20, 32, 33). Hydrolysis of ATP, and hence conversion of Hsp70 to the ADP-bound state, is stimulated by substrate binding and by cochaperones of the Hsp40/DnaJ family (6, 20, 25) through events that are likely to occur in a synergistic manner (16). At physiologically relevant Hsp40-Hsp70 ratios (<0.1), this synergy may ensure that substrates first bind to Hsp70 and subsequently become tightly associated with the ADP-bound high-affinity state of Hsp70, so that refolding of denatured substrates is promoted (16). In vitro data also show that as Hsp40-Hsp70 ratios increase, the rate of ATP hydrolysis by Hsp70 accelerates, and this correlates with less efficient refolding of denatured substrates (16).

Consistent with these in vitro data, we demonstrated that overexpression of Hsp40 in mammalian cells (yielding high Hsp40-Hsp70 ratios) reduces refolding of the in situ heat-inactivated reporter protein firefly luciferase (23). In contrast, when Hsp40 is overexpressed together with Hsp70, an enhancement of refolding of luciferase is observed. These experiments raise the surprising possibility that, under stressful conditions, the maximal refolding activity of Hsp70 may require events that occur in the ATP-bound conformation, perhaps by taking advantage of the high substrate on-rate under these conditions, and that rapid conversion to the ADP-bound conformation may actually be deleterious to proper refolding, at least under some circumstances. However, this hypothesis has not been tested formally.

Two other cochaperones, Hip and Bag-1, act on the chaperone activity of Hsp70 in the ADP-bound state, with antagonistic effects on the refolding kinetics of the bound substrate. Both cochaperones bind to the ATPase domain of Hsp70 and enhance (Hip) or inhibit (Bag-1) protein refolding of Hsp70-bound substrates in vitro (4, 10, 12, 19, 35, 36). Recently, we demonstrated that increased expression of Hip in mammalian cells similarly enhances the refolding of the heat-inactivated reporter enzyme luciferase (30). Hip also protects luciferase from irreversible denaturation under conditions of ATP depletion, as would be expected based on in vitro assays of Hip function. In contrast to the effects of Hip, Bag-1 inhibits Hsp70 chaperone activity in mammalian cells at physiologically relevant concentrations (29), again confirming in vitro observations of the activity of this cochaperone. These studies also demonstrate the utility of measuring luciferase folding in cells as a means to elucidate the function of cochaperones in a cellular environment.

Yet another regulator of the Hsp70 chaperone, CHIP, was recently identified, and its effects on Hsp70 have been charac-

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terized in vitro (2). CHIP is a 35-kDa protein that interacts with the carboxyl terminus of Hsp70 through its amino-terminal domain, which contains three tetratricopeptide repeats (TPR) and an adjacent charged domain. Two different groups have shown that CHIP, similar to Hsp40, acts on Hsp70 in the ATP-bound state; however, in contrast to Hsp40, CHIP inhibits the Hsp40-stimulated ATPase activity of Hsp70 (2, 21). In parallel, CHIP inhibits the refolding of thermally denatured firefly luciferase in vitro in the presence of Hsp70 and Hsp40 at a molar CHIP to Hsp40 to Hsp70 ratio of 2:2:1 (2), although its effects on refolding in mammalian cells have not yet been tested.

In addition to the TPR domain, CHIP also contains a U-box (a modified RING finger) at its carboxyl terminus, which suggests a role in ubiquitin-dependent protein degradation (1, 15). Indeed, overexpression of CHIP in mammalian cells enhances the degradation of the cystic fibrosis transmembrane-conductance regulator (CFTR) (21), and recently CHIP has been shown to be a bona fide E3-ubiquitin ligase (13). However, it must be noted that the effect of CHIP as an enhancer of (Hsp70-dependent) protein degradation is substrate specific, as maturation and degradation of proteins other than CFTR (such as the transferrin receptor and apolipoprotein B48) are unaffected by CHIP overexpression (21). Both the U-box domain and the TPR domain of CHIP are essential for CFTR degradation (21). The U-box is, however, dispensable for the ability of CHIP to inhibit Hsp70 ATPase activity in vitro (21), suggesting that the degradative effects of CHIP are not caused simply by a nonspecific inhibition of CHIP on the protein-folding function of Hsp70.

The effect of CHIP on CFTR degradation seems to depend only on interaction with Hsp70 and not, for example, with Hsp40 or Hsp90 (21). In addition, the degradation-promoting activity is specific for the cochaperone CHIP, as increased expression of Hip, Hsp40, or Bag-1 has no effect on CFTR degradation (21), although overexpression of Bag-1 in CHIP-overexpressing cells may enhance the degradation effect of CHIP (8). CHIP also interacts with the TPR acceptor site of Hsp90, which leads to remodeling of the Hsp90 complex (7). As such, CHIP abolishes the steroid binding activity and transactivation potential of the glucocorticoid receptor by enhancing glucocorticoid receptor ubiquitination and degradation via the proteasome (7). This action also requires both the TPR domain of CHIP and its U-box domain, suggesting that the effect of CHIP on glucocorticoid receptor degradation also may be uncoupled from the effects of CHIP on the protein-folding function of Hsp70.

We have investigated the in situ effects of full-length CHIP, a TPR deletion mutant, and a U-box deletion mutant on the protein-refolding function of Hsp70. To this end, different CHIP variants were coexpressed with firefly luciferase in hamster fibroblasts, either alone or combined with Hsp70, Hsp40, and/or Bag-1. Especially at relatively high CHIP-Hsp70 expression ratios and in contrast to our expectations, CHIP attenuated the rate of thermal inactivation of luciferase and increased the rate and extent of luciferase refolding at a permissive temperature. This action required the TRP domain of CHIP. The U-box domain, however, was dispensable for this action. Coexpression of Hsp40 acted competitively with the effect of CHIP on the rate of luciferase inactivation, and dom-

inant-negative constructs of Hsp40 (22) completely abolished the effects of CHIP on protein folding, indicating that this activity of CHIP is Hsp70 dependent. In contrast, coexpression of Bag-1 did not influence the effect of CHIP on thermal inactivation of luciferase, although it did inhibit the refolding kinetics, as found previously (29, 30). These studies thus provide a basis for understanding the importance of the ATP hydrolysis step in the Hsp70 chaperone cycle in preventing irreversible protein unfolding under conditions of cellular stress and emphasize the need to understand both in vitro and cellular functions of chaperones and cochaperones in order to assign cellular functions to components of the protein-folding machinery.

MATERIALS AND METHODS

Plasmids and constructs. pRSVLL/V encodes cytoplasm-localized luciferase under the control of a Rous sarcoma virus (RSV) long terminal repeat (24). In some experiments, we used pRSVLL/V-EGFP, which encodes luciferase tagged at its C terminus with the enhanced green fluorescent protein (EGFP). In biochemical experiments, luciferase and luciferase-EGFP behave in a similar manner (data not shown). For expression of CHIP, we used pCDNA3-CHIP, pCDNA3-CHIP-Myc (Myc tagged), pCDNA3-CHIP-ΔTRP (residues 32 to 145 deleted), and pCDNA3-CHIP-ΔE4 (residues 196 to 303 deleted) (7). Plasmids pCMV70, pCMV40, pCMV40-1-75 (residues 76 to 340 deleted), and pCDNA-Bag-1M were used to express Hsp70 (human, inducible form), Hsp40 (also known as Hdj-1) (22, 23), and the murine 29-kDa isoform of Bag-1, respectively (4, 29). As a control, we always used the empty vector pSP64 (Promega).

Cell culture, transfections, stress treatments, and luciferase measurements. Hamster lung fibroblasts (O23) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco, Paisley, United Kingdom). Transient transfections with luciferase and (co)chaperone-encoding plasmids were performed with Lipofectamine according to the manufacturer's instructions (Gibco, Paisley, United Kingdom). The plasmid quantity was kept equal to 10 μg/25-cm² dish by the addition of plasmid pSP64 (Promega), usually with 1 μg of DNA of each relevant plasmid per dish, unless otherwise indicated. At 48 h after transfection, the medium was replaced with medium containing 20 μg of cycloheximide per ml and 20 mM MOPS, pH 7.0. After a 30-min incubation, cells were heated to inactivate the luciferase. Subsequently, the cells were reincubated at 37°C to allow reactivation of the luciferase.

For ATP depletion experiments, Chinese hamster ovary (CHO) cells grown in Ham's F12 medium supplemented with 10% fetal bovine serum were transferred to tissue culture tubes and incubated at 37°C in glucose-free Dulbecco's modified Eagle's medium in the presence of 3% fetal bovine serum, 10 mM 2-deoxyglucose, and 20 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) (18). During this incubation, triplicate samples were taken for measurement of luciferase activity.

To test for effects of (co)chaperone expression on luciferase turnover rates, cells were incubated in the presence of cycloheximide without heating for up to 6 h at 37°C. At various time points, triplicate samples were taken for the measurement of luciferase activity as previously described (24).

Western blot analysis and immunofluorescence analysis. Cells were trypsinized, resuspended in phosphate-buffered saline, and lysed by addition of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and sonication prior to SDS-PAGE and Western blot analysis. CHIP was detected with polyclonal antiserum raised against the full-length CHIP protein (2). Hsp70 was detected with a monoclonal antibody specific for the heat-inducible form of Hsp70 (C92; Stressgen). After incubation with appropriate secondary antibodies, specific binding was visualized by enhanced chemiluminescence (ECL; Amersham). To measure luciferase solubility, cells were separated into Triton X-100-soluble and Triton X-100-insoluble fractions as described before (29), and the samples were loaded on SDS–10% polyacrylamide gels. Since the commercially available antibodies against luciferase are of rather poor quality, luciferase-EGFP was used in these experiments. A monoclonal antibody directed against EGFP (8363-2; Clontech) was used for detection of the fusion protein. For intracellular localization, we used confocal microscopy (Leika TCS SP2).

Pulse labeling of newly synthesized proteins. CHO cells in 10-cm plates were transfected with a plasmid expressing a Myc-tagged Hsc70 with or without pCDNA3-CHIP, as described before (7). At 48 h after transfection, the cells

were incubated in medium containing [^{35}S]methionine (Amersham, SJ1015) for 10 min at 37°C. The medium was removed, and the cells were immediately lysed in radioimmunoprecipitation assay (RIPA) buffer containing either apyrase (10 U/ml) or ATP (2.5 mM) plus an ATP-regenerating system on ice. Equal amounts of lysates were immunoprecipitated with an anti-Myc antibody (Santa Cruz) under these conditions, and labeled proteins were separated by SDS-PAGE, followed by autoradiography. For quantification, the amount of nascent polypeptides coimmunoprecipitating with Myc-tagged Hsc70 was measured with a phosphorimager (Molecular Dynamics 445 SI).

RESULTS

Luciferase degradation is not affected by CHIP and Hsp70 overexpression. In vitro, luciferase is an efficient substrate for Hsp70, and its refolding by Hsp70 can be influenced by CHIP (2). In cells, CHIP can target at least one Hsp70 substrate (CFTR) for proteosome-dependent degradation (21). In addition, CHIP has been implicated in the ubiquitination of heat-denatured luciferase in vitro (27). We therefore first tested whether the rate of luciferase degradation in cells is also influenced by CHIP. To determine luciferase turnover at 37°C under conditions in which denaturation of luciferase is minimal, we coexpressed luciferase with CHIP, Hsp70, or both. At 48 h after transfection, the cells were treated with cycloheximide to inhibit new protein synthesis. Luciferase activity was monitored for up to 6 h. Under these conditions, luciferase activity decreased to about 45% of the control in 6 h, indicating that luciferase has a half-life of about 5.5 h (Fig. 1A). The coexpression of CHIP or Hsp70 alone or both combined had no effect on the half-life of luciferase, indicating that neither protein affects the rate of luciferase degradation in the absence of thermal stress. In addition, the specific activity of luciferase was not significantly affected by coexpression of CHIP or Hsp70 at this temperature (not shown).

We next considered whether CHIP and/or Hsp70 might preferentially affect the rate of degradation of luciferase when it has been heat denatured. To test this possibility, transfected cells were exposed to heat shock at 45°C for 30 min (conditions which result in denaturation and inactivation of the majority of luciferase), and the total amount of luciferase was determined by Western blotting. No loss of luciferase signal was seen immediately after (H) or 3 h after (H+R) heat shock, irrespective of overexpression of Hsp70, CHIP, or both (Fig. 1B). Also, we did not observe the accumulation of slowly migrating forms of luciferase, which would have been indicative of (chaperone-dependent) ubiquitination.

Endogenously, CHIP localizes to the endoplasmic reticulum of mammalian cells but is also present diffusely in the cytosol (21). To ascertain that the absence of an effect of ectopically expressed CHIP on protein degradation is not due to an abnormal intracellular localization, we performed confocal microscopic analysis. As can be seen in Fig. 2, a significant fraction of ectopically expressed CHIP localized to the cytosol (Fig. 2A and E), where most of the EGFP-tagged luciferase also resided both before and after heat shock (Fig. 2B and F). Taken together, and in contrast to the situation in vitro, our observations indicate that luciferase is not a (major) substrate for the ubiquitin ligase activity of CHIP in cells, either at 37°C or when unfolded by heat shock.

Overexpression of CHIP enhances refolding of heat-denatured luciferase. Because luciferase is a thermolabile reporter
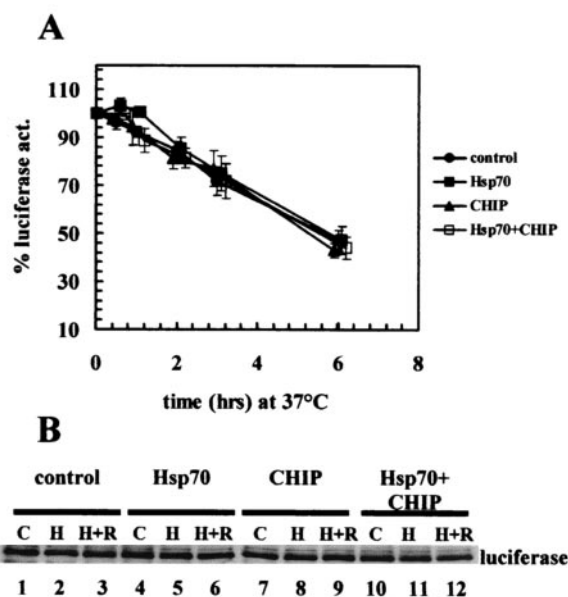

FIG. 1. Luciferase degradation and CHIP overexpression. (A) O23 cells were transfected with Hsp70 (solid squares), CHIP (solid triangles) CHIP and Hsp70 (open squares), or a control plasmid (solid circles) together with pRSVLL/V, encoding firefly luciferase targeted to the cytoplasm. At 48 h after transfection, the cells were treated with cycloheximide (CHX) to inhibit new luciferase synthesis, and luciferase activity was measured and plotted relative to the activity prior to cycloheximide treatment (=100%). Data are means ± standard errors of the means from three to four independent experiments performed in quadruplicate. (B) O23 cells were transfected with Hsp70 (lanes 4 to 6), CHIP (lanes 7 to 9), CHIP and Hsp70 (lanes 10 to 12), or a control plasmid (lanes 1 to 3) together with pRSVLL/V, encoding firefly luciferase targeted to the cytoplasm. At 48 h after transfection, the cells were lysed either immediately (C), directly after a 30-min heat shock at 45°C (H), or 180 min at 37°C after the heat shock (H+R), and luciferase expression was measured by Western blotting.

protein, measurement of its activity and solubility has proven to be extremely helpful in unraveling the ability of Hsp70 cochaperones to modify the refolding of denatured proteins in mammalian cells in situ (22, 23, 28–30). With this approach, CHIP was transiently coexpressed (Fig. 3A, lane 1) with cytoplasm-localized luciferase in O23 hamster fibroblasts, which constitutively express Hsc70 but not Hsp70. At 48 h after transfection, the cells were heated for 30 min at 45°C to inactivate luciferase. Cycloheximide was added prior to heat shock to prevent new protein synthesis during postheat incubation at 37°C to be able to specifically monitor chaperone-dependent refolding of luciferase. In contrast to what can be expected from the in vitro data (2), overexpression of CHIP alone led to a fivefold increase in luciferase refolding within 60 min compared to effects observed in control O23 cells (Fig. 3B). Similar effects were detected in CHO cells (data not shown).

CHIP overexpression did not cause a general stress response in the O23 cells used here, as indicated by the fact that we found no alterations in expression levels of stress-inducible Hsps such as Hsc70, Hsp70, Hsp90, and Hsp40 within this time frame (data not shown). This is consistent with previous observations in MEK293 cells (21) and excludes the possibility that the observed effects of CHIP on protein refolding are due
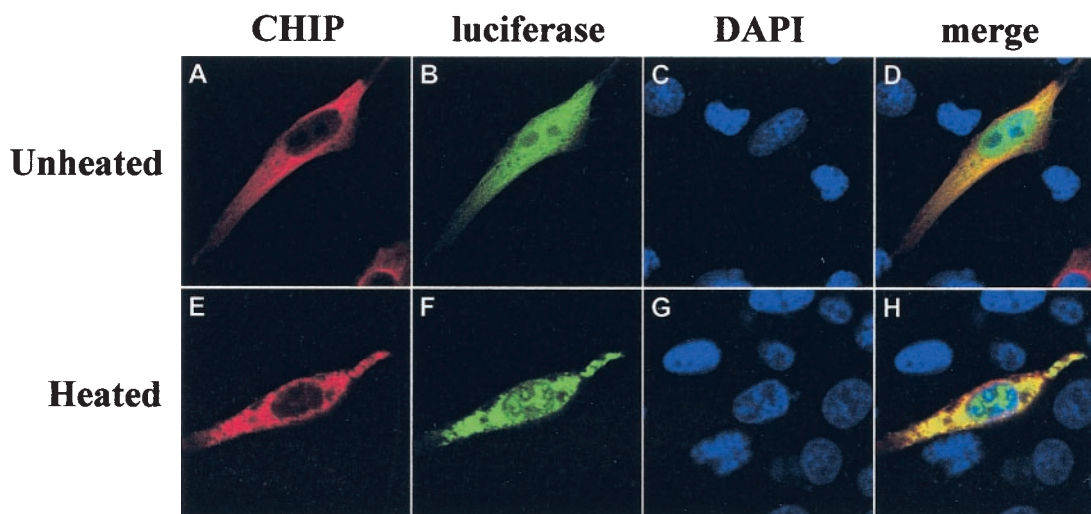
FIG. 2. Overexpressed CHIP shows partial colocalization with cytosolic luciferase before and after heat shock. O23 cells were transfected with CHIP and cytoplasmic firefly luciferase-encoding constructs. At 48 h after transfection, the cells were processed for immunohistochemistry and subsequent confocal microscopic analysis to detect the localization of ectopically expressed CHIP (A and E) and luciferase (B and F) under control (A to D) and heat shock (E to H) conditions. The cells were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) (C and G). Merged images are shown in panels D and H.

to global alterations in steady-state levels of Hsps under these conditions. Overexpression of Hsp70 is also effective in enhancing luciferase refolding in the cytosol (22, 23, 28) (Fig. 3B). When CHIP was coexpressed with Hsp70, the luciferase refolding was not significantly different from that in cells expressing Hsp70 alone (Fig. 3B), suggesting that the effects of CHIP on refolding are dependent on the stoichiometry between CHIP and Hsc70/Hsp70.

Deletion of the U-box domain (residues 190 to 303; Fig. 3A, lane 3), which is required for CHIP-dependent CFTR degradation (21), had no effect on the ability of CHIP to refold luciferase (Fig. 3C), consistent with previous observations that the U-box is not required for CHIP-dependent modification of Hsp70 ATPase and chaperone activity in vitro (21). In contrast, deletion of the TPR domain (CHIPΔTPR: deletion of residues 32 to 145; Fig. 3A, lane 2, and Fig. 3C) abolished the ability of CHIP to enhance refolding in cells, indicating that the effect of CHIP depended on interactions with either Hsc70 or Hsp90. To distinguish between these two possibilities, cells were treated with geldanamycin (1 μg/ml), a drug that displaces ATP from Hsp90 chaperone complexes, hence impairing its chaperone activity (31, 34). Geldanamycin reduced the extent of refolding in control cells (Fig. 3D), indicating that disruption of the endogenous Hsp90 chaperone network antagonizes the cellular capacity to refold heat-denatured luciferase in mammalian cells. Expression of CHIP, however, enhanced refolding in the geldanamycin-treated cells to a similar extent as in nontreated cells (Fig. 3D). These observations indicate that Hsp90 activity is not needed for the observed effects of CHIP and imply that enhanced luciferase refolding by CHIP is probably independent of its ability to bind Hsp90 and alter its activity.

Enhanced protein refolding by CHIP requires a functional Hsc/Hsp70 chaperone machine. Previously, we have demonstrated that expression of a truncated form of Hsp40 (Hsp40-J; residues 76 to 340 deleted) has a dominant negative effect on the chaperone activity of Hsc70/Hsp70 (22). To test whether the effects of CHIP on luciferase refolding are dependent on Hsp70 function, we coexpressed CHIP and Hsp40-J with luciferase in O23 cells. If the action of CHIP is independent of the action of Hsc70/Hsp70, coexpression Hsp40-J expression should result in levels of refolding comparable to that seen in cells transfected with CHIP alone. However, this was not the case; when CHIP was coexpressed with Hsp40-J, refolding of luciferase returned to control levels (Fig. 3E). Hsp40-J expression alone resulted in levels of refolding even below control (not shown). Taken together with the preceding experiments, we can conclude that the effects of CHIP on luciferase refolding require a functional Hsc70/Hsp70 chaperone machine, and are independent of Hsp90 activity.

Competition between effects of Hsp40 and CHIP on luciferase inactivation. The observed CHIP-mediated enhancement of the Hsc70/Hsp70-dependent chaperone capacity seems unexpected considering the inhibitory action of CHIP on the ATPase activity of Hsc70/Hsp70. However, we have previously found that expression of Hsp40 at levels sufficient to obtain a relatively high ratio of Hsp40 to Hsc70/Hsp70 in cells has negative effects on cellular protein refolding (23). Under these same conditions, the rate of luciferase inactivation during heat shock was enhanced (23). As Hsp40 participates in loading substrates onto Hsp70, one might explain this negative effect on refolding by arguing that high Hsp40:Hsp70 ratios in cells might have changed the equilibrium between Hsp70-bound and Hsp40-bound (non-refolding competent) substrates. However, overexpression of Hsp40 containing a single point mutation in the HPD motif of its J-domain (Hsp40 H/Q [28]), which lacks the capacity to interact with Hsp70 family members (37) but has an intact substrate binding domain (17), has no effect on protein inactivation and refolding in cells (22; data not shown). Therefore, the negative effect of Hsp40 on
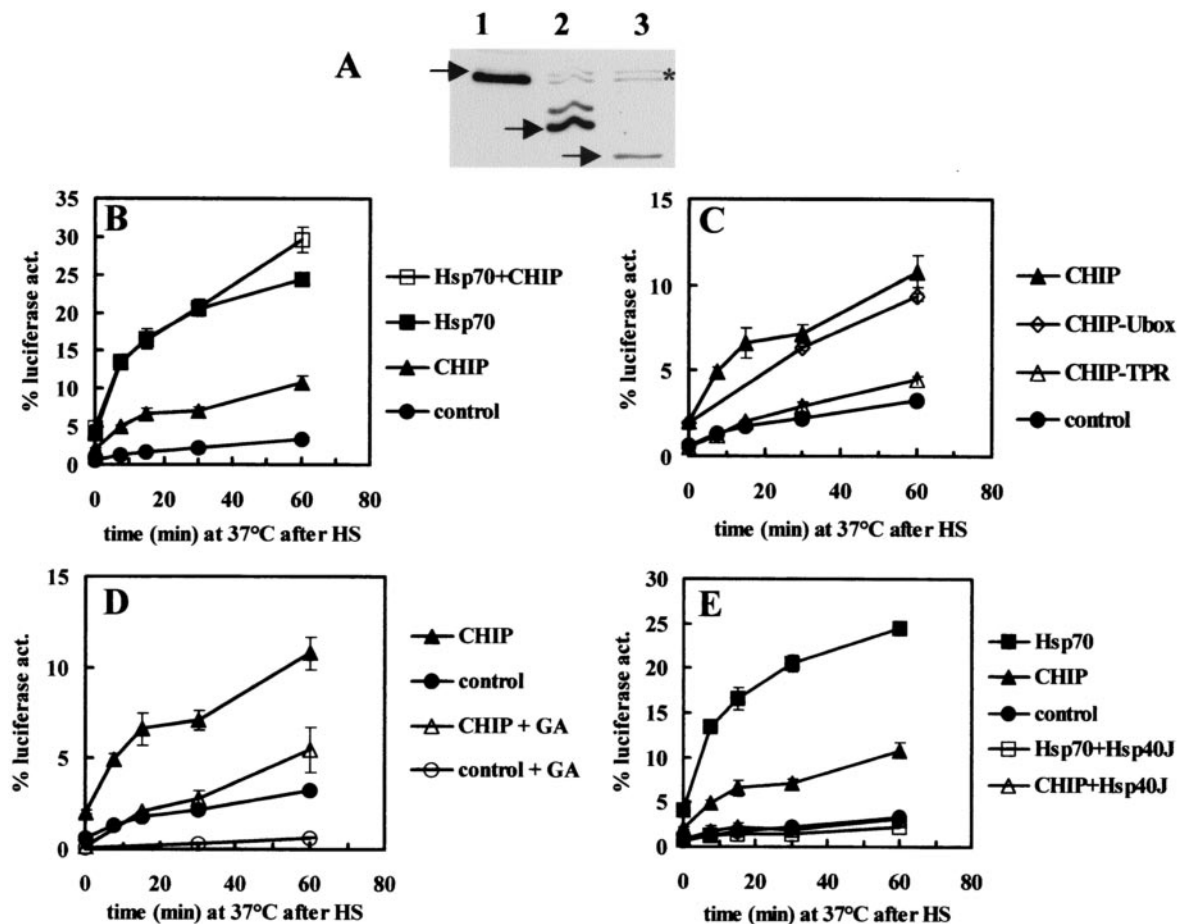
FIG. 3. Enhanced refolding of heat-denatured luciferase by CHIP is independent of Hsp90 function but dependent on interaction with and functional activity of Hsp70. O23 cells were transiently transfected with various chaperone-encoding constructs together with pRSVLL/V, encoding firefly luciferase targeted to the cytoplasm. At 48 h after transfection, the cells were treated with cycloheximide to inhibit new luciferase synthesis, heated for 30 min at 45°C, and reincubated for 0 to 60 min at 37°C to allow luciferase refolding. Samples were taken at the indicated time points, and luciferase activity was measured and plotted relative to the activity prior to treatment (=100%). Data are means ± standard errors of the means from 3 to 10 independent experiments performed in quadruplicate. (A) Expression levels of full-length (lane 1, arrow) and mutant CHIP (lane 2, CHIPΔTPR, arrow; lane 3, CHIPΔUbox, arrow) after transient transfection. Endogenous CHIP expression is marked with an asterisk; (B) Luciferase refolding in cells expressing CHIP alone (solid triangles), Hsp70 alone (solid squares), or Hsp70 and CHIP (open squares) compared to cells transfected with an empty vector (control, solid circles). (C) Luciferase refolding in cells overexpressing full-length CHIP (solid triangles) compared to CHIP lacking the U-box domain (CHIPΔUbox; open diamonds), CHIP lacking its TPR domain (CHPΔTPR; open triangles), and cells transfected with an empty vector (control; solid circles). (D) Luciferase refolding in cells overexpressing CHIP (triangles) compared to cells transfected with an empty vector (control; circles) in the absence (solid symbols) or presence of the Hsp90-disrupting agent geldanamycin (GA; 1 μg/ml added 10 min prior to the heat shock) (open symbols). (E) Luciferase refolding in cells overexpressing CHIP (triangles) or Hsp70 (squares) either alone (open symbols) or when cotransfected with the J-domain of Hsp40 (solid symbols). Cells were (co)transfected with 1:1 ratios of the plasmids encoding the respective (co)chaperones.

folding is most likely explained by its ability to enhance the ATPase activity of Hsp70. Since CHIP has the opposite effect on Hsp70 (i.e., inhibiting rather than enhancing the ATPase activity of Hsp70), one may envision that Hsp40 and CHIP have competitive effects on Hsp70-dependent protein refolding.

As a first step to test this model, we examined the effects of CHIP or CHIPΔTPR on the rate of luciferase inactivation in cells. Indeed, when cells were heated at either 42°C (Fig. 4A) or 45°C (Fig. 4B), the rate of luciferase inactivation was retarded by CHIP but not by CHIPΔTPR. This protective effect of CHIP was about 50% of the protection seen in cells overexpressing Hsp70 (Fig. 4A and B). Similar observations were

made for luciferase solubility. In unheated control cells, about 10% of the luciferase is recovered in the Triton X-100-insoluble pellet fraction and 90% was soluble, irrespective of chaperone overexpression (Fig. 4C, lanes 1 to 9). After heating, more than 85% of the luciferase ended up in the pellet fraction of control cells (Fig. 4C, lanes 10 to 12). In cells overexpressing Hsp70, more luciferase remained soluble and only ≈50% appeared in the pellet (Fig. 4C, lanes 16 to 18). In cells overexpressing CHIP, protection against luciferase insolubility was also observed; only about 60% of the luciferase ended up in the pellet under these conditions (Fig. 4C, lanes 13 to 15). These results, when considered in the context of the sensitizing effect of Hsp40 on the rate of luciferase inactivation during heat
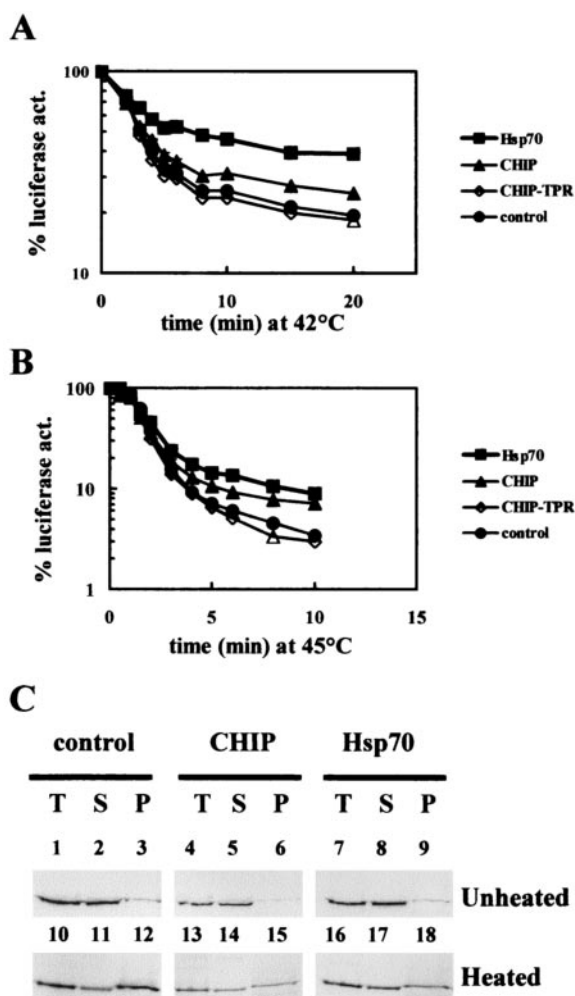
FIG. 4. Thermal inactivation and insolubilization of luciferase is retarded in CHIP-overexpressing cells. O23 cells were transiently transfected with constructs expressing Hsp70 (solid squares), CHIP (solid triangles), CHIP lacking the TPR domain (CHIPΔTPR; open triangles), or an empty vector (control; solid circles) together with constructs encoding (EGFP-tagged) firefly luciferase targeted to the cytoplasm. At 48 h after transfection, the cells were heated at either 42°C (A) or 45°C (B). Samples were taken at the indicated time points during heating, and luciferase activity was measured and plotted relative to the activity prior to treatment (=100%). Data are from a typical experiment performed in quadruplicate. In panel C, unheated and heated (30 min at 42°C) samples from control cells (lanes 1 to 3 and 10 to 12, respectively), CHIP-overexpressing cells (lanes 4 to 6 and 13 to 15, respectively), and Hsp70-overexpressing cells (lanes 7 to 9 and 16 to 18, respectively) were fractionated, and total cell lysates (T), the Triton X-100-soluble (S), and insoluble (P) fractions were loaded on SDS-polyacrylamide gels. Luciferase-EGFP was detected with EGFP antibodies. Data are from a typical experiment.

shock (23), suggest that CHIP and Hsp40 may be competitive in terms of affecting the chaperone activity of Hsp70.

To assess this hypothesis more directly, we transfected cells with different ratios of plasmids encoding CHIP and Hsp40 together with luciferase. These cells were heated for 30 min at 45°C and assayed for luciferase activity either immediately (Fig. 5A and B), after 1 h (Fig. 5C and D), or 3 h at 37°C (Fig. 5E and F). It is clear from these data that Hsp40 and CHIP

acted competitively in terms of luciferase inactivation, insofar as increasing concentrations of CHIP blocked Hsp40-dependent augmentation of luciferase inactivation and increasing concentrations of Hsp40 antagonized the ability of CHIP to suppress luciferase inactivation (Fig. 5A). In addition, the extent of luciferase reactivation during recovery was similarly affected (Fig. 5C and E). The level of inactivation after heat shock closely correlates with the yield of refolding during recovery, further supporting the notion that the effects of CHIP, like those of Hsp40, are mediated via regulation of Hsc70/Hsp70 (and not Hsp90) by altering its ability to bind to proteins during heat shock and maintain them in a refolding-competent form. When CHIP was coexpressed with Bag-1, no competition on the extent of protein inactivation was observed (Fig. 5B). Consistent with the known effect of Bag-1 to influence protein refolding via an effect on the Hsp70 in the ADP-bound state (4, 10, 19, 29, 30, 35, 36), Bag-1 did inhibit the yield of refolding after heat shock (Fig. 5D and F).

Effects of Hsp70 and CHIP under conditions of cellular ATP depletion. We previously showed that ATP depletion can result in inactivation of luciferase, an effect that can be attenuated by Hsp70 or Hip overexpression, but not by overexpression of Hsp40 (30). These findings are consistent with the presumed actions of Hsp40 and Hip derived from in vitro studies. As most of the available Hsp70 will be in the ADP-bound state under conditions of ATP depletion, Hip, which regulates the ADP-bound conformation of Hsp70, can still affect Hsp70 chaperone activity. In contrast, Hsp40, which acts on Hsp70 in the ATP-bound state, can no longer influence the Hsp70 chaperone activity under these circumstances. By analogy, we hypothesized that CHIP also should have no effect on the rate of inactivation of luciferase under conditions of ATP depletion if its effects were fully dependent on Hsp70 and acting to modulate the ratio of Hsp70 that is in the ATP- rather than ADP-bound conformation.

To test this model, we used CHO cells, as they are more sensitive to ATP depletion-mediated proteotoxicity than are hamster lung fibroblasts. CHO cells were depleted of ATP with 2-deoxyglucose plus CCCP, which results in a decline of total cellular ATP to less than 2% after 60 min, irrespective of chaperone overexpression (30). Whereas the rate of luciferase inactivation caused by ATP depletion was attenuated by overexpression of Hsp70, CHIP had no effect under these conditions (Fig. 6). Similar results were obtained in H9c2 cells, which are also sensitive to ATP depletion by 2-deoxyglucose and CCCP (14). These data also indicate that the effects of CHIP cannot be explained by global effects such as, for example, increasing the free pool of Hsp70 available for stress-related chaperone activities or altering the balance between degradative and refolding chaperone functions, since such global effects should result in to protective effects even under conditions of ATP depletion.

CHIP enhances Hsc70 association with nascent polypeptide chains in the presence of an ATP-regenerating system. To explore further whether CHIP affects the ability of Hsc70/Hsp70 to interact with nonnative proteins in an ATP- or ADP-dependent manner, we tested the ability of CHIP to influence the binding of Hsc70 to nascent polypeptide chains. This also allowed us to determine whether such effects would have an impact on naturally occurring substrates at physiological tem-
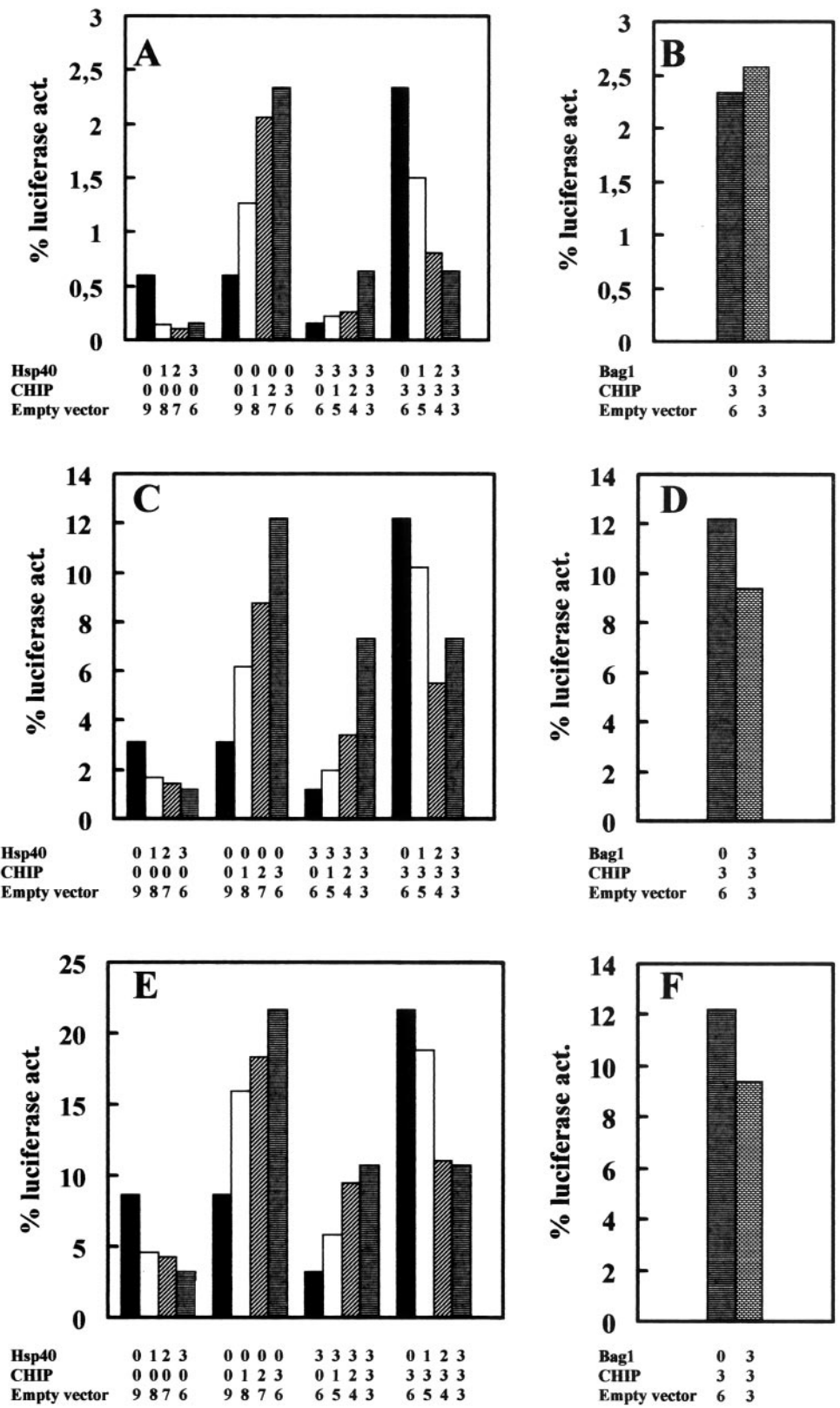
FIG. 5. CHIP acts competitively with Hsp40 but not Bag-1 in affecting luciferase denaturation and refolding. O23 cells were transiently transfected with different ratios of plasmids encoding Hsp40, CHIP, and an empty vector (A, C, and E) or at a 1:1 ratio with a Bag-1-expressing plasmid (B, D, and F) together with 1 μg of pRSVLL/V, encoding firefly luciferase targeted to the cytoplasm. At 48 h after transfection, the cells were heated for 30 min at 45°C, and samples were taken immediately after heating (A and B) or 1 h (C and D) or 3 h (E and F) after heating for measurement of luciferase activity. The activity data are plotted relative to the activity prior to treatment (=100%). Data are means from three to four independent experiments performed in quadruplicate. Error bars were omitted for reasons of clarity but never exceeded 10% of the average value.
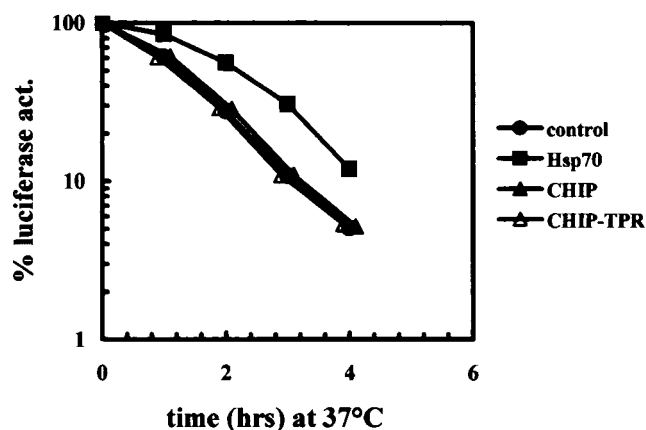
FIG. 6. Rate of inactivation of luciferase due to ATP depletion is not retarded by CHIP. CHO cells were transiently transfected with constructs expressing Hsp70 (solid squares), CHIP (solid triangles), CHIP lacking the TPR domain (CHIPΔTPR; open triangles), or an empty vector (control; solid circles) together with pRSVLL/V, encoding firefly luciferase targeted to the cytoplasm. At 48 h after transfection, the cells were treated with CCCP in medium containing 2-deoxyglucose, which reduces cellular ATP levels to below 2% within 1 h. Samples were taken at the indicated time points during heating, and luciferase activity was measured and plotted relative to the activity prior to treatment (=100%). Data are means ± standard error of the mean from three independent experiments performed in quadruplicate.

peratures. To this end, Myc-tagged Hsc70 was cotransfected with or without CHIP in CHO cells. 48 h after transfection, the cells were pulse-labeled with [^{35}S]methionine for 10 min to label nascent chains. Cell lysates were prepared in the presence of apyrase or an ATP-regenerating system and Myc-Hsc70 and coimmunoprecipitating labeled proteins were pulled down with an anti-Myc antibody.

In lysates treated with apyrase, there was an abundance of labeled nascent proteins that coimmunoprecipitated with Myc-Hsc70 (Fig. 7, lanes 1 and 3), consistent with the ability of Hsc70 to bind to nascent chains (3). No coimmunoprecipitation was found in cells without Myc-Hsp70 expression (data not shown). CHIP had no effect on the extent of coimmunoprecipitation under this condition (Fig. 7, lane 2, and Table 1), which is concordant with the absence of an effect of CHIP on luciferase inactivation during ATP depletion. In the presence of the regenerating system, allowing active cycling of Hsc70 and hence binding and release of substrates, the amount of labeled nascent protein that coimmunoprecipitated with Myc-Hsp70 was drastically reduced, roughly equivalent to background levels (Fig. 7, lanes 4 and 6, compared with lanes 1 and 3). In cells overexpressing CHIP, however, a threefold increase in the amount of Hsc70-associated nascent proteins was found (Fig. 7, lane 5, and Table 1).

To exclude the possibility that this increase was due to binding of nascent chains to CHIP directly rather than an CHIP-mediated enhancement of substrate binding to Hsp70, cells were transfected with a CHIP deletion mutant lacking a functional TPR domain so that it cannot bind to Hsp70. No coimmunoprecipitation of nascent chains with CHIP was found (data not shown). These results are consistent with the hypothesis that CHIP, by slowing down the Hsp70 cycle, can enhance
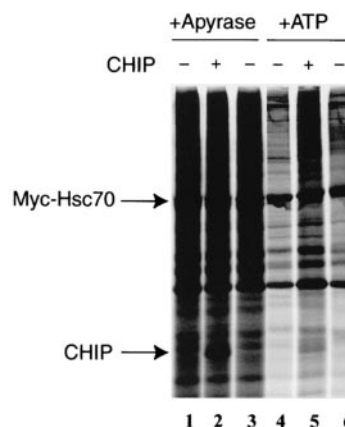

FIG. 7. CHIP enhances the amount of nascent polypeptide chains that coimmunoprecipitate with Hsp70 in the presence of an ATP-regenerating system. CHO cells were transfected with Myc-tagged Hsc70 with (+) or without (−) CHIP and pulse-labeled with [^{35}S]methionine at 48 h after transfection. Lysates were prepared in the presence of apyrase (lanes 1 to 3) or an ATP-regenerating (ATP) system (lanes 4 to 6). Immunoprecipitation was performed with Myc-Hsp70, and the precipitated material was run on polyacrylamide-SDS gels. Arrows indicate the positions of Hsc70 and CHIP. Data are from a typical experiment (see Table 1 for quantitative data).

the loading of substrates onto Hsp70. Finally, similar experiments were performed cotransfecting either Hsp40 or Bag-1 together with Myc-tagged Hsc70 (Table 1). As with its effects on luciferase refolding, Hsp40 had the opposite effect compared to CHIP on the amount of nascent chains coimmunoprecipitating with Hsp70: in the presence of ATP (but not in the absence of ATP), the fraction of Hsp70-bound nascent chains was reduced by Hsp40. Bag-1 had no significant effect on nascent chain binding of Hsp70 when ATP was present but significantly reduced the fraction of Hsp70-bound nascent chains when the experiments were carried out under ATP-depleted conditions, consistent with its effect as a negative regulator of Hsp70 via an effect on the Hsp70 in the ADP-bound state (4, 10, 19, 29, 30, 35, 36).

TABLE 1. CHIP enhances and Hsp40 reduces the amount of nascent polypeptide chains that coimmunoprecipitate with Hsp70 in the presence of an ATP-regenerating system[a]

Transfection	Relative amt of nascent chains coimmunoprecipitating with Hsp70 ± SEM	
	Without ATP (apyrase)	With ATP
None (control)	1.00 ± 0.08	1.00 ± 0.20
CHIP	1.04 ± 0.14	3.21 ± 0.63
Hsp40	1.15 ± 0.21	0.63 ± 0.11
Bag-1	0.30 ± 0.18	0.80 ± 0.16

[a] CHO cells were transfected with Myc-tagged Hsc70 alone or together with CHIP, Hsp40, or Bag-1 and pulse-labeled with [^{35}S]methionine at 48 h after transfection. Lysates were prepared in the presence of apyrase or an ATP-regenerating (ATP) system. Immunoprecipitation was performed with Myc-Hsp70, the precipitated material was run on SDS-PAGE, and the amount of radioactivity was quantified with a phosphorimager. Averaged data from three to four independent experiments are provided.

DISCUSSION

The activity of mammalian Hsp70 is influenced by a variety of regulators that can determine the ability of the protein to act as a molecular chaperone in protein refolding assays in vitro. In addition to the DnaJ family proteins such as Hdj-1/Hsp40, which can stimulate the activity of the cytoplasmic Hsp70 chaperones and thereby enhance the ability of Hsp70 to assist in refolding of proteins (6, 25), three other classes of proteins have been identified that interact with Hsp70 to modulate the Hsp70 reaction cycle. Hip, initially identified in a protein interaction trap assay with the Hsp70 ATPase domain, forms a complex that enhances Hsp70 chaperone activity in vitro by stabilizing the ADP-bound conformation of the chaperone (12). The family of Bag-1 proteins can also associate with the Hsp70 ATPase domain, yet they inhibit the Hsp70-dependent refolding of proteins in vitro by facilitating nucleotide exchange (4, 10, 19, 35, 36). With our luciferase-based in situ model, we have been able to demonstrate similar cofactor dependence of Hsp70-mediated refolding reactions in the mammalian cytosol and nucleus: overexpression of Hsp40 (together with Hsp70 overexpression) and Hip enhanced luciferase refolding in cells (22, 23, 30), whereas overexpression of Bag-1 inhibited this (29, 30). Unexpectedly, however, we now show that overexpression of CHIP, an inhibitor of the Hsp70 ATPase and chaperone activity in vitro, resulted in enhanced protein refolding in mammalian cells in situ.

CHIP and protein degradation. Based on the data presented here, the statement (21) that CHIP is a cochaperone that converts Hsc70 from a folding machinery into a degradation factor seems not to be generally true for all Hsp70 substrates. Although overexpression of CHIP in mammalian cells enhances the degradation of CFTR, no effect of CHIP on the stability of the transferrin receptor or apolipoprotein B48 (21) or luciferase (this report) has been observed. It therefore remains unclear what determines the fate (refolding or degradation) of Hsp70-bound substrates. In any case, the degradation-related properties of CHIP can be clearly dissected from its ability to affect Hsp70-dependent protein refolding. Whereas both the U-box domain and the TPR domain of CHIP are essential for CFTR degradation (21), the U-box is not required for the ability of CHIP to inhibit Hsp70 ATPase activity in vitro (21) and for the effects of CHIP on refolding luciferase in mammalian cells (this report).

Effects of CHIP on refolding unfolded substrates are dependent on functional Hsp70. Our observation that the effects of CHIP are dependent on the presence of the TPR-domain, which is required for CHIP-Hsp70 interactions (21) (2, 7), suggests that the influence of CHIP on refolding is due to direct modulation of the activity of Hsp70. Yet one could envision that CHIP overexpression evokes a stress response in cells leading to an elevation in the level of Hsp70, thereby increasing the cells' chaperone capacity. However, in the O23 cells used here and under the experimental conditions used, we found elevation neither in Hsp70 levels nor in the level of any other main cytosolic chaperones. Similar results were obtained when overexpressing CHIP in MEK293 cells (21). Therefore, the observed effects of CHIP cannot be explained by altering the steady-state levels of endogenous heat shock proteins in these experiments. Also, the effects of CHIP are not likely to

be related to altered free pools of Hsp70 under these conditions, as no protection of CHIP overexpression was seen against denaturation by ATP, a stress that can still be modulated by altered Hsp70 levels (18, 30; this report).

The TPR domains in CHIP are also involved in the interaction of CHIP with Hsp90 to remodel the Hsp90 complex (7). As a result of this remodeling, one can envision that elevated ratios of CHIP increase the level of free chaperones (Hsp70 or/and Hsp90) available for refolding heat-denatured substrates. Therefore, we treated cells with the drug geldanamycin, which is capable of disrupting the Hsp90 complex (31, 34). We found that treatment of cells with geldanamycin, by itself, inhibited refolding in the cytosol. To avoid potential nonspecific effects of geldanamycin, the concentrations of the drug used were kept low (1 μg/ml) and the incubation was short; hence, they are indicative of some role for constitutively expressed Hsp90 in refolding heat-denatured luciferase. Moreover, treatment with geldanamycin did not affect the ability of CHIP-overexpression to enhance luciferase refolding. These data demonstrate that the CHIP-dependent effects observed in these studies are not mediated via Hsp90. Finally, the ability of dominant negative Hsp40 constructs, which block the function of Hsp70, to abrogate CHIP-dependent refolding (Fig. 2), strongly supports the contention that the effects of CHIP are dependent on direct functional interactions with Hsp70.

Reconciliation of Hsp70 modulation by CHIP for in vitro and cellular refolding activities. It should be emphasized that our cell data are fully consistent with the mechanism of action of CHIP on Hsp70 proposed on the basis of in vitro data. The action of CHIP results in effects on luciferase refolding opposite to those observed with Hsp40, consistent with their respective and opposing effects on Hsp70 ATPase activity. In addition, CHIP does not enhance the chaperone capacity of cells under conditions of ATP-depletion. Similarly, CHIP does not affect the binding of nascent chains by Hsp70 under conditions where no ATP is present. CHIP can increase the fraction of nascent chains coimmunoprecipitating with Hsc70 only when sufficient ATP is present. The cell data presented here are therefore in complete agreement with the in vitro data in that CHIP exerts is effects by acting on the ATP-bound state of Hsp70 and by decreasing the rate of ATP hydrolysis (2). However, whereas the mechanism of CHIP's effects on Hsp70 function is consistent in in vitro and cellular assays, the consequences of this mechanism differ. In in vitro studies, inhibition of Hsp70 ATPase activity by CHIP reduces Hsp70-dependent refolding, whereas overexpression of CHIP in cells leads to a higher yield of luciferase refolding.

The question remains how to reconcile our observation that CHIP, an inhibitor of the forward reaction of the Hsp70 cycle (both in vitro and in cells), enhances protein refolding in cells. Several laboratories have shown that Hsp40 participates in vitro with Hsp70 in folding of luciferase in a substoichiometric concentration (16, 20, 25). However, if the molar ratio of Hsp40 to Hsp70 is raised above 1:1, the folding reaction is inhibited (16), possibly because (excess) Hsp40 will reduce Hsp70 recycling. As a result, substrates will remain bound to Hsp70 in the ADP-bound state for a longer time and no new substrates can be captured. Consequently, the pool of Hsp70 that can bind substrates is more rapidly depleted. Especially at limiting levels of Hsp70, these effects should be most dramatic.

Indeed, our previous (23) and present studies show a negative effect on folding when enhancing the forward Hsp70 reaction by overexpression of Hsp40 alone (but not the mutant Hsp40 H/Q, which cannot interact with Hsp70).

One explanation for the effects of CHIP is that CHIP increases Hsp70 folding activity through a functional interference with the interaction between Hsp40 and Hsp70. Thus, under conditions in which rapid cycling can occur (i.e., in cells in the presence of ATP), CHIP may merely ameliorate the consequences of the functional access of Hsp40 to Hsp70 in a competitive manner, thereby counteracting the effect of Hsp40 on Hsp70 availability. Alternatively, overexpression of CHIP could have enhanced the likelihood of substrate binding by Hsp70 in cells by increasing the fraction of Hsp70 in the ATP-bound form. The ATP-bound form of Hsp70, has a higher substrate on-rate (9, 20, 32, 33). Although the off-rate of substrates from Hsp70 is even higher when it is in the ATP-bound state (i.e., lower substrate affinity), attenuating forward cycling at this step of hydrolysis by CHIP could lead to a better loading of denaturing substrates to Hsp70. Again, this would have the highest impact at limiting levels of Hsp70 and it would be consistent with the negative effect on folding that is observed when enhancing the forward Hsp70 cycle by excess Hsp40 in vitro (16) and in cells (23; this report) and the competition between Hsp40 and CHIP, as reported here. We cannot conclude which of these two explanations for the effects of overexpressing CHIP in cells is the most probable one, as this highly depends on which of the reactions (ATP hydrolysis or substrate dissociation) is the fastest in living cells, something we cannot measure at present.

As the endogenous expression of CHIP is extremely variable across cell types and since levels of CHIP in cell types such as skeletal muscle are 20- to 30-fold higher than those observed in cell types such as CHO or COS (C. Patterson, unpublished observations), our observations, although obtained with transient transfection experiments yielding relatively high levels of overexpression, likely have relevance for protein folding reactions under physiological conditions. In support of this, it was recently found that cells deficient in CHIP (derived from CHIP knockout mice) are more susceptible to L-canavanine and heat shock (Q. Dai and C. Patterson, unpublished data).

With the current data, combined with those published previously (22, 23, 28–30), we have characterized the cellular action of the four regulators of the Hsp70 chaperone cycle known to date. Results from these studies are sometimes consistent with in vitro observations (e.g., the effects of Hip and Bag-1) and are sometimes counterintuitive (e.g., the effects of CHIP and Hsp40, especially at low Hsp70 concentrations). The cumulative results of these studies indicate both the strengths and the weaknesses of in vitro assays of chaperone function; in particular, these studies suggest that in vitro observations underestimate the effects of dynamic changes in Hsp70 conformation that occur in an endogenous milieu of chaperones, cochaperones, and substrates. In addition, our findings in cells argue that the role of the ATP-bound conformation of chaperones may be far more important in the refolding cycle than has been estimated by in vitro studies (in which the ATP-bound conformation is generally considered a quiescent, nonpermissive state), particularly under conditions of cell stress, in which nucleotide cycling may be rapid and inefficient.

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