THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 7, Issue of February 16, pp. 4677–4682, 2001

Printed in U.S.A.

Modulation of *in Vivo* HSP70 Chaperone Activity by Hip and Bag-1*

Received for publication, October 25, 2000, and in revised form, November 9, 2000
Published, JBC Papers in Press, November 13, 2000, DOI 10.1074/jbc.M009745200

Ellen A. A. Nollen‡, Alexander E. Kabakov§, Jeanette F. Brunsting‡, Bart Kanon‡, Jörg Höhfeld¶, and Harm H. Kampinga‡‖

From the ‡Department of Radiation and Stress Cell Biology, Faculty of Medical Sciences, University of Groningen, 9713 AV Groningen, The Netherlands, §Medical Radiology Research Center, Obninsk 249020, Russia, and ¶Institute for Cell Biology, Rheinische Friedrich-Wilhelms-Universität Bonn, D-53121 Bonn, Germany

The chaperone activity of Hsp70 is influenced by the activities of both positive and negative regulatory proteins. In this study, we provide first time evidence for the stimulating effect of the Hsp70-interacting protein Hip on the chaperone activity in the mammalian cytosol. Overexpressing Hip enhances the refolding of the heat-inactivated reporter enzyme luciferase expressed in hamster lung fibroblasts. Also, it protects luciferase from irreversible denaturation under conditions of ATP depletion. We demonstrate that these stimulating actions depend on both the presence of the central Hsp70-binding site and the amino-terminal homo-oligomerization domain of Hip. The carboxyl terminus (amino acids 257–368) comprising the 7 GGMP repeats (Hsc70-like domain) and the Sti1p-like domain are dispensable for the Hip-mediated stimulation of the cellular chaperone activity. Bag-1, which inhibits the Hsp70 chaperone activity both *in vitro* and *in vivo,* was found to compete with the stimulatory action of Hip. In cells overexpressing both Hip and Bag-1, the inhibitory effects of Bag-1 were found to be dominant. Our results reveal that *in vivo* a complex level of regulation of the cellular chaperone activity exists that not only depends on the concentration of Hsp70 but also on the concentration, affinity, and intracellular localization of positive and negative co-regulators. As the Hsp70 chaperone machine is also protective in the absence of ATP, our data also demonstrate that cycling between an ATP/ADP-bound state is not absolutely required for the Hsp70 chaperone machine to be active *in vivo*.

Molecular chaperones of the Hsp70 family play a key role in the control of protein folding during protein biogenesis, protein transport through membranes, and when cells are exposed to proteotoxic stress (1–3). Hsp70 chaperones have a specialized domain capable of binding exposed hydrophobic stretches in polypeptide chains, which would otherwise aggregate within the densely packed environment of the cell. Substrate binding and release is coupled to a cycle of ATP binding and hydrolysis by Hsp70. *In vitro*, a variety of regulatory proteins of this Hsp70 cycle have been identified that influence the properties of the Hsp70 chaperone in protein refolding.

Proteins of the Hsp40/DNA-J family can increase the Hsp70 chaperone activity by enhancing the ATPase activity of Hsp70 (4, 5). This action is dependent on the interaction of the J-domain of Hsp40 with the ATP binding domain of Hsp70, but it also requires the interaction between the extreme carboxyl-terminal EEVD sequence of Hsp70 and the carboxyl-terminal domain of Hsp40. By using a luciferase reporter assay in mammalian cells, we demonstrated that Hsp40 *in vivo* also stimulates Hsp70-mediated refolding (6). In fact, by using dominant negative Hsp40 mutants, we found that the Hsp70 chaperone activity was completely inhibited, thereby proving that interaction with J-domain-containing proteins is required for the *in vivo* refolding activity of Hsp70 (7).

The homo-oligomeric protein Hip cooperates with Hsp70 in protein folding by stabilizing the ADP-bound state of Hsp70. Hip directly binds to the ATPase domain of Hsp70 when it is converted to the ADP-bound state by proteins of the Hsp40 family (8). The Hsp70-binding site of Hip consists of multiple tetratricopeptide repeats and a flanking charged α-helix. The extreme amino terminus contains a domain required for Hip oligomerization. As monomeric Hip still can bind to Hsp70, homo-oligomerization is not required for Hsp70 interaction (9). Besides affecting the Hsp70 chaperone cycle *in vitro*, Hip alone can also bind to unfolded proteins and prevent their aggregation. Yet refolding of proteins to their active state requires cooperation with other chaperones (8, 10). The rat Hip protein shows >90% identity at the amino acid level with p48, a protein that is involved in the maturation of the human progesterone receptor (8, 11).

In addition to these positive regulators of the Hsp70 chaperone two inhibitory proteins, CHIP and Bag-1, have been identified *in vitro*. CHIP, also a tetratricopeptide repeat-containing protein, inhibits the ATPase activity of Hsp70 (12) and prevents the formation of stable Hsp70-substrate interaction required for proper refolding. Bag-1, originally discovered as a Bcl-2-associated protein (13), is expressed in various isoforms, which originate from different translation initiation sites, all of which can inhibit Hsp70 refolding activity both *in vitro* (14–16) and *in vivo* (17). Recently several additional Bag-1-like proteins have been identified (18). All Bag-1-like proteins share a conserved 40–50-amino acid "BAG" domain and compete with Hip in binding to the ATPase domain of Hsp70 *in vitro* (14–16, 18).

Here we investigated whether Hip and Bag-1 affect Hsp70 chaperone activity in hamster fibroblasts and Chinese hamster ovary cells. In both these cell lines, Hsc70 is constitutively expressed, whereas Hsp70 is only expressed upon exposure of the cells to stress. Overexpressed Hip exclusively localized to the cytoplasm and enhanced refolding of heat-inactivated cytoplasmic luciferase and protected it from inactivation during

* This work was supported by the Dutch Cancer Society NKB Grant 95-1082 (to H. H. K.), a grant from the Netherlands Science Organization (NWO) (to A. E. K and H. H. K.), and a grant from the German Science Foundation (to J. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed: Dept. of Radiation and Stress Cell Biology, Faculty of Medical Sciences, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands. Tel.: 31-50-3632903-2911; Fax: 31-50-3632913; E-mail: h.h.kampinga@med.rug.nl.

ATP depletion. For both activities the presence of the Hsp70-binding site as well as the oligomerization domain was required. Hip was active when overexpressed alone as well as when overexpressed together with Hsp70 suggesting that it stimulates the action of both Hsc70 and Hsp70. As demonstrated before (17), Bag-1 inhibited Hsp70 chaperone function. In cells overexpressing Hip and Bag-1, the inhibitory effects of Bag-1 were dominant. The results reveal that *in vivo* a complex level of regulation of the Hsp70 chaperone activity exists. Furthermore, they show that cycling between an ATP/ADP-bound state is not absolutely required for the Hsp70 chaperone machine to be active.

MATERIALS AND METHODS

Plasmids and Constructs—pRSVLL/V encodes cytoplasmic luciferase under control of a Rous sarcoma virus-long terminal repeat promoter (provided by Dr. S. Subramani, University of California, San Diego). PSBC/Hip and pSBC vectors containing the various Hip mutants were created by ligation of an *Eco*RI fragment from pGAD10-Hip behind the SV40 promoter in pSBC-1 (provided by Dr. H. Hauser, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany). Hip and Hip mutants were inserted into pCDNA-3/HA[1] after polymerase chain reaction amplification resulting in an in-frame fusion of a triple hemagglutinin tag.

pCDNA/HA-BAG-1 and pCDNA/HA-BAG-1ΔC were created by cloning an *Eco*RI-*Xho*I fragment from pGEX-4T-1/BAG-1 and pGEX-4T-1/BAG-1ΔC (14) behind a cytomegalovirus promoter in pCDNA-3/HA (provided by Dr. S. Ness, Northwestern University, Evanston, IL), respectively (17).

pCMV40 consists of a *Hin*dIII-*Bam*HI fragment encoding the cDNA sequence of human Hsp40 (19) inserted into the *Bgl*II-*Hin*dIII sites of the eukaryotic expression vector. Plasmid pCMV70 was constructed by inserting a *Hin*dIII fragment encoding the cDNA for Hsp70.1, the human-inducible DnaK homologue (20) into the *Hin*dIII site of pCMV5 (6).

Cell Culture and Transfections—OT70 cells are hamster lung fibroblasts (O23) in which Hsp70 expression can be controlled by the tetracycline-responsive transactivator (tTA) expression system (21). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 1 mg/ml G418 (Life Technologies, Inc.), 1 mg/ml hygromycin (Roche Molecular Biochemicals), and 3 μg/ml tetracycline (Sigma). G418 and hygromycin were absent during all experiments.

For ATP depletion experiments Chinese hamster ovary cells (CHO) were used as they were more sensitive to ATP depletion-mediated proteotoxicity than the hamster lung fibroblast. The CHO cells were grown in Ham's F-12 medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.).

For both cell lines, transient transfections were performed using LipofectAMINE according to the procedure of the manufacturer (Life Technologies, Inc.).

Stress Treatments and Measurement of Luciferase Activity—For the heat shock experiments, OT70 cells were transiently transfected with pRSVLL/V and co-chaperone-encoding plasmids or pCDNA as a control. 24 h after transfection, the cells were transferred into tissue culture tubes in medium with or without 3 μg/ml tetracycline for induction of Hsp70 expression. Another 24 h later, the medium was replaced with medium containing 20 μg/ml cycloheximide and 20 mM MOPS, pH 7.0. After a 30-min preincubation, cells were heated for 30 min at 45 °C to inactivate luciferase. Subsequently, the cells were reincubated at 37 °C to allow for reactivation of luciferase. At various time points, triplicate samples were taken for the measurements of luciferase activity as described before (22).

For the ATP depletion experiments, CHO cells were transiently transfected with pRSVLL/V and co-chaperone-encoding plasmids or pCDNA as a control. 24 or 48 h after transfection, the cells were transferred into 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 (2-DG), and 20 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (23). During this incubation triplicate samples were taken for measurement of luciferase activity.

Western Blot Analysis and Immunofluorescence Analysis—For Western blot analysis the cells were trypsinized, resuspended in phosphate-buffered saline, lysed by addition of SDS-polyacrylamide gel electrophoresis sample buffer, and sonicated prior to SDS/Western blot analysis. The HA-tagged Hip and HA-tagged BAG-1 proteins were detected using a monoclonal HA antibody (provided by Dr. R. Lamb, Northwestern University, Evanston, IL). Hsp70 was detected by C92, a monoclonal antibody specific for the heat-inducible form of Hsp70 (Stressgen).

Indirect immunofluorescence was performed as described previously (6). OT70 cells transfected with pCDNA/HA-Hip or pCDNA/HA-BAG-1 were immunostained with the mouse monoclonal anti-HA tag antibody and an fluorescein isothiocyanate-labeled anti-mouse secondary antibody (Sigma). The images were made by confocal laser scanning microscopy (Zeiss).

Measurement of Cellular ATP Levels—Determination of the relative ATP content in cell lysates was based on the same measurement of ATP-dependent luciferase activity (22), whereas exogenous luciferin and luciferase but not ATP were added to the lysates (24). The light emission was measured on a luminometer for 10 s after mixing the cell lysate aliquots (0.15 ml) with 0.1 ml of a lysing buffer containing 5 μg/ml luciferase, 0.3 mM luciferin, 0.3 mM AMP, and 1 mM dithiothreitol. The ATP level in untreated cells was considered as 100%. Care was taken to use the same amount of cells (10^5) in the same volume of a lysing buffer.

RESULTS

Overexpression of Hip Enhances Chaperone Capacity—A biochemical assay that allows the measurement of cellular chaperone activity in both the cytoplasmic and nuclear compartment of mammalian cells has been previously established (22). This assay has allowed us to delineate the *in vivo* chaperone activity of Hsp70 and modulation thereof by Hsp40/HdJ-1 (6, 7, 21) and Bag-1 (17). In this model luciferase is expressed in the nucleus or cytoplasm of hamster cells that are subsequently heated, after which activity of the enzyme is measured at various time points after heat shock.

HA-tagged Hip was transiently expressed in OT70 hamster fibroblasts in which only Hsc70 and not Hsp70 is constitutively expressed and in which Hsp70 expression can be regulated by the tetracycline-responsive system (21). Expression of HA-Hip did not influence basal Hsp70 nor did it affect TET-inducible Hsp70 expression (Fig. 1*A*), as was shown before (8). Hip was exclusively present in the cytoplasm (Fig. 1*B*) and did not change its localization upon heat shock (data not shown). In the absence of Hsp70 expression, overexpression of Hip resulted in an increase in the refolding of the cytoplasmic luciferase (Fig. 1*C*). When overexpressed together with Hsp70, Hip also enhanced the refolding of cytoplasmic luciferase to levels above that for expression of Hsp70 alone (Fig. 1*D*). Consistent with the cytoplasmic localization of Hip, no effect of Hip has been detected on the inactivation and recovery of nuclear luciferase (data not shown).

To establish the mode of action of Hip-mediated enhancement of the cellular chaperone capacity, we expressed a variety of mutant variants that were previously characterized *in vitro* in terms of their ability to bind to Hsc/Hsp70 and their ability to oligomerize (Fig. 2, *A* and *B* (9)). None of the expressed mutants resulted in an induction of Hsp70, indicating that they did not cause a stress response in the cells. Also, they did not affect the level of induction of Hsp70 by tetracycline removal (data not shown), and all mutant proteins were expressed to equal levels as the wild-type proteins as detected by an HA antibody (Fig. 2*B*). The Hip mutant with a truncation of the homo-oligomerization domain but containing the entire Hsp70 binding domain (Hip-(15–368)) was not capable of stimulating luciferase refolding after heat shock like wild-type Hip alone (Fig. 2*C*) and also could not stimulate refolding in the presence of Hsp70 (Fig. 2*D*). This implies that oligomerization of Hip is

[1] The abbreviations used are: HA, hemagglutinin; CHO, Chinese hamster ovary; MOPS, 4-morpholinepropanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; 2-DG, 2-deoxyglucose.

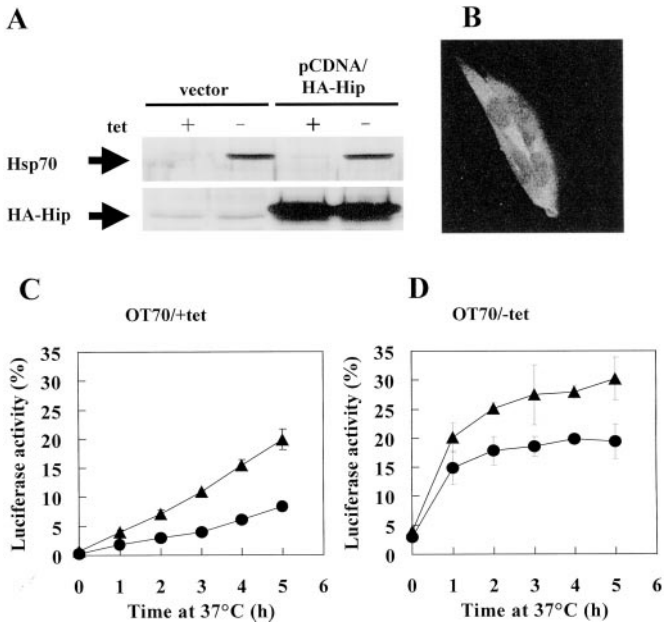

FIG. 1. **Expression of Hip in OT70 enhances Hsp70-mediated reactivation of heat-denatured firefly luciferase.** OT70 cells were transiently transfected with pCytluc (encoding cytoplasmic luciferase) together with pCDNA (vector) or pCDNA/Hip and grown in medium with or without tetracycline (*tet*) for induction of Hsp70 expression. *A,* Western blot analysis of Hsp70 and Hip expression. *B,* immunofluorescent analysis of HA-Hip localization after staining the transfected cells with a monoclonal HA antibody. *C* and *D,* cells transfected with pCDNA (vector: *circles*) or pCDNA/Hip (*triangles*) and grown in medium with (*C*) or without (*D*) tetracycline for regulated induction of Hsp70 expression were pretreated with cycloheximide (20 μg/ml) and heated for 30 min at 45 °C to inactivate luciferase. After heating, cells were allowed a recovery period of 0–5 h at 37 °C, and samples for luciferase activity were taken at the indicated time points. Data points represent the mean of 3 independent experiments and error bars indicate S.E. of the means.

required for its enhancement of the cellular chaperone activity. Expression of the mutant Hip that lacks part of or the entire Hsp70 binding domain but contains the amino-terminal oligomerization domain (Hip-(1–218)) was also incapable of stimulating protein folding in the cell (Fig. 2, *C* and *D*). Thus, the observed enhanced refolding under the condition of Hip overexpression seems to depend on the interaction with Hsp70 or Hsc70 (when the tetracycline system is not induced). Expression of the Hip-(110–368) protein, lacking both the Hsp70 binding and oligomerization capacity was also ineffective in stimulating Hsp70-mediated recovery (Fig. 2, *C* and *D*). However, the mutants Hip-(1–303) and Hip-(1–257) were as effective as the full-length Hip in enhancing luciferase refolding (Fig. 2, *C* and *D*). This indicates that the last 110 carboxyl-terminal amino acids (amino acids 258–368) are not essential in for the effects of Hip.

Effects of Hsp70 and Hip Under Conditions of ATP Depletion—By having established that Hip can stimulate Hsp70-mediated refolding of heat-inactivated luciferase, we wanted to gain further insight in the mechanism of this effect. *In vitro* data have shown that Hip results in the stabilization of the Hsp70-ADP substrate complex (8). To test this *in vivo* we decided to investigate the effect of Hsp70 and Hip in cells after ATP depletion. Such treatments were shown to be proteotoxic and capable of inducing protein inactivation and aggregation, including inactivation and insolubilization of the reporter enzyme firefly luciferase (24–26). Here, ATP depletion in CHO cells was accomplished by treatment with 10 mM 2-deoxyglucose + 20 μM CCCP in the glucose-free medium. This treatment results in a rapid decline of total cellular ATP to less than 2% after 60 min (Fig. 3*A*). Overexpression of chaperones had no effect of the kinetics of ATP depletion (data not shown).

As a result of the ATP depletion luciferase activity gradually declined in the cells, with kinetics about 4-fold slower than that for the ATP depletion (Fig. 3*A*). In contrast to the situation after heat shock where luciferase can be refolded from the heat-denatured state when the heat treatment is not severe or when chaperones are overexpressed, no significant luciferase refolding was observed upon ATP replenishment under any of the conditions tested (data not shown). Remarkably, when the ATP-dependent Hsp70 chaperone was overexpressed, the rate of luciferase denaturation during ATP depletion was clearly attenuated. Hsp40/Hdj-1 overexpression alone or combined with Hsp70 had no effect on the rate of luciferase inactivation during ATP depletion (Fig. 3*B*). The latter is consistent with the presumed mechanistic action of Hsp40, *i.e.* stimulation of the conversion of the ATP-bound state of Hsp70 to an ADP-bound state by accelerating ATP hydrolysis (4, 5).

Like for heat shock, full-length Hip was capable of enhancing the cellular chaperone activity under conditions of ATP depletion. Luciferase inactivation was retarded in cells overexpressing Hip; this action was again dependent on the ability of Hip to oligomerize and to bind to Hsp/Hsc70 as mutants lacking the respective domains were incapable of demonstrating chaperone activity (Fig. 3*C*). Combining Hip and Hsp70 overexpression led to a moderate further protection compared with either Hip or Hsp70 overexpression alone (data not shown).

Bag-1 Suppresses Hip-mediated Enhancement of the Hsp70 Chaperone Function—We have previously shown that various isoforms of the Bag-1 protein can inhibit the *in vivo* chaperone action of Hsp70 at physiologically relevant, stoichiometric levels (17). *In vitro* data have indicated that both Hip and Bag-1 compete for interaction with the ATPase domain of Hsp70 *in vitro* (14–16, 18).

Therefore, we transiently coexpressed Bag-1 (29-kDa isoform) and Hip, either alone or jointly in luciferase expressing OT70 cells, and we examined the effects of Hsp70-mediated reactivation of heat-inactivated luciferase. As demonstrated before (17) the 29-kDa Bag-1 predominantly localized in the nucleus but also could be clearly detected in the cytoplasm (Fig. 4*A*). Both Hip and Bag-1 were HA-tagged and expressed to equal total levels in OT70 cells (Fig. 4*B*). Considering the exclusive cytoplasmic localization of Hip (Fig. 1*B*), we therefore conclude that its cytoplasmic concentration is higher than the cytoplasmic concentration of Bag-1. The HA-Bag-1, although predominantly nuclear, was capable of substantially inhibiting the Hsp70-mediated recovery of cytoplasmic luciferase. Surprisingly, coexpression of Bag-1 and Hip led to one-half the reactivation of the cytoplasmic luciferase compared with overexpression of Hip and Hsp70 alone (Fig. 3*C*). This suggests that the inhibitory effect of Bag-1 is dominant to the stimulatory effect of Hip. The inhibitory effect of Bag-1 requires its carboxyl-terminal Hsp70 binding domain as overexpression of Bag-ΔC did not inhibit the stimulatory effects of Hip (data not shown).

DISCUSSION

In vitro experiments have identified several partner proteins of Hsp70 that regulate its function as a molecular chaperone. These include both positive regulators (Hsp40/Hdj1 and Hip) and negative regulators (CHIP, Bag-1) (4, 8, 12, 14–16, 18). One valid issue is whether *in vitro* chaperone-mediated protein refolding studies accurately reflect *in vivo* events given the high cellular concentration of proteins, the vast excess of potential substrates, and that each cellular compartment has multiple chaperones. Here, we have focused on the *in vivo* action of Hip, a member of a distinct family of Hsp70 co-

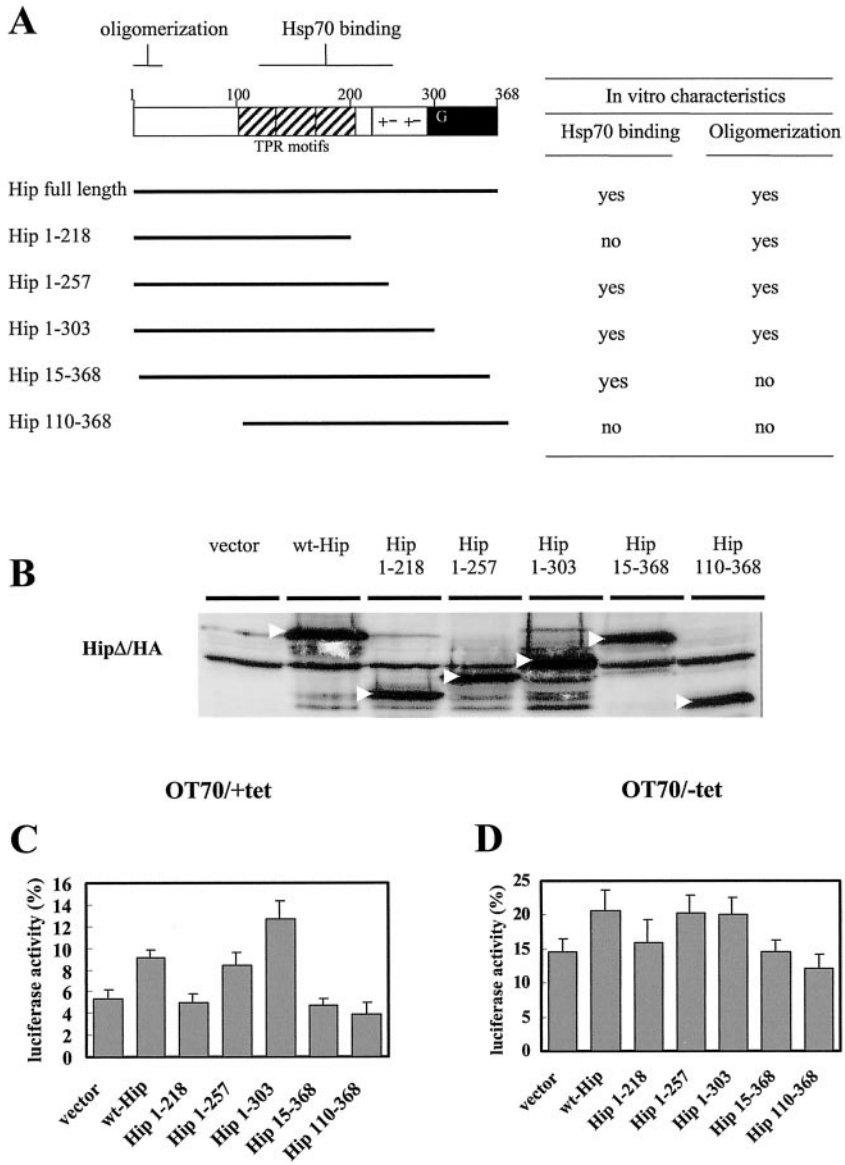

FIG. 2. **The Hsp70 binding domain and oligomerization domain of Hip are required for enhancement of Hsp70-mediated reactivation of heat-denatured firefly luciferase.** OT70 cells were transiently transfected with pCytluc (encoding cytoplasmic luciferase) together with pCDNA (vector) or the indicated full-length (*wt*) or mutant Hip cDNAs and grown in medium with or without tetracycline (*tet*) for induction of Hsp70 expression. *A,* constructs used and their *in vitro* characteristics (after Irmer and Hohfeld (9)). *B,* Western blot analysis of mutant Hip expression (HA-Hip bands are indicated by *white arrowheads*). *C* and *D,* transfected cells were pretreated with cycloheximide (20 μg/ml) and heated for 30 min at 45 °C to inactivate luciferase. After heating, cells were allowed a recovery period of 3 h at 37 °C, and samples for luciferase activity were taken at the indicated time points. Data points represent the mean of 3 independent experiments, and *error bars* indicate S.E. of the means.

chaperones that interacts with the Hsp70 ATPase domain and enhances folding of denatured luciferase *in vitro* (8, 27).

In vivo, Hip is a component of steroid hormone receptor complexes and is also involved in the assembly of Hsp70 into multichaperone complexes with Hsp90 (11, 28). A role for Hip as a positive regulator of Hsp70 activity *in vivo* had not been demonstrated previously. Both constitutively expressed Hip as well as overexpressed Hip was found to be exclusively located in the cytoplasm (Ref. 8 and this report). This localization did not change during or after heat shock (not shown), unlike Hsp70 and Hsp40 that change their localization from mostly cytoplasmic before to merely nuclear after heat shock (29–32). Consistent with its cytoplasmic localization, Hip enhanced refolding of the cytoplasmic but not nuclear luciferase. The effect was already seen when Hip was overexpressed alone, and co-expression with Hsp70 merely resulted in additive enhancement of luciferase refolding.

In vitro, Hip was shown to be capable of binding directly to unfolded proteins and to prevent their aggregation (8, 10). As such, one could interpret the increase in cellular chaperone activity by Hip overexpression independent from Hsp70. However, deletion of the Hsp70-binding site of Hip without affecting its homo-oligomerization domain resulted in the loss of a Hip effect on luciferase refolding after heat shock. Although this mutant was never tested for its ability to prevent aggregation *in vitro*, the most likely explanation for observed effects of Hip in mammalian cells is that Hip requires a direct interaction with Hsp70 consistent with the situation *in vitro* (8, 10). Furthermore, this is supported by the finding that Bag-1, which competes with Hip for Hsp70 binding (see also below), inhibited the Hip-mediated increment in cellular chaperone activity. Since Hip also stimulated refolding in the absence of Hsp70 expression, it can be deduced that Hip is at least equally effective in stimulating refolding by Hsc70, which is constitutively expressed in the OT70 cells.

By using a series of other Hip mutants, it was demonstrated that Hip oligomerization is also required for its action as a co-chaperone of Hsp70/Hsc70, whereas the last 110 carboxyl-terminal amino acids are not essential for this function. The amino acids 278–311 of Hip contain 7 imperfect GGMP repeats that are found in most cytoplasmic Hsc70s but not in Hsp70s (33). The function of this motif is yet unclear, but from our *in vivo* data as well as from *in vitro* data (9) it can be deduced that it is not involved in regulating Hsp70 chaperone activity. Furthermore, our data indicate that the very carboxyl-terminal domain (amino acids 316–368), which is structurally related to the Hop homologues Sti1p and human IEF SSP3521, is not required for regulating Hsp70 refolding activity.

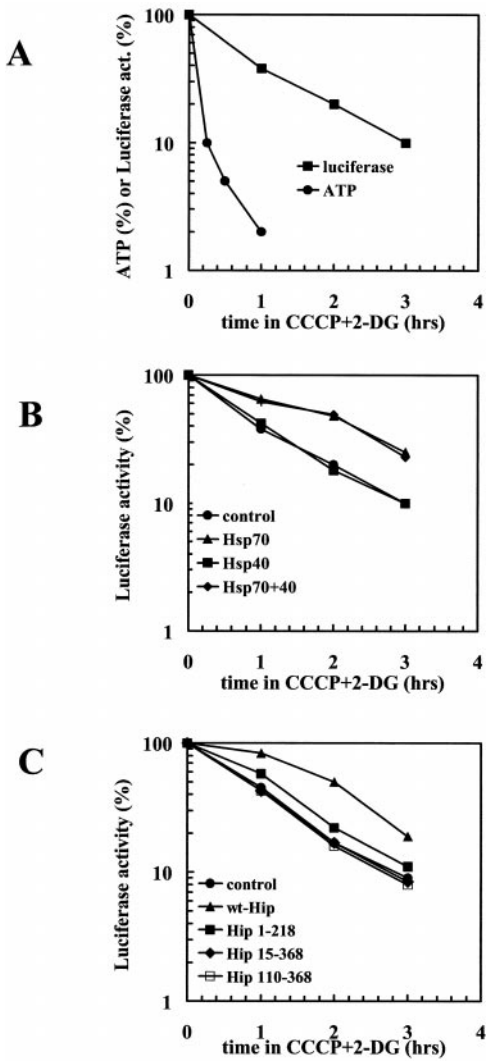

FIG. 3. **Hsp70-mediated protection of luciferase inactivation during ATP depletion: effects of Hip.** CHO cells were transiently transfected with pCytluc (encoding cytoplasmic luciferase) alone or together with cDNAs encoding the various (co)chaperones and treated with CCCP + 2-DG 48 h after transfection. *A,* kinetics of ATP depletion (*circles*) and luciferase inactivation (*squares*) during treatment with CCCP + 2-DG. *B,* effects of overexpression of Hsp70 (*triangles*), Hsp40 (*squares*), or Hsp70 + Hsp40 (*diamonds*) on luciferase inactivation during ATP depletion (*circles*: vector only). *C,* effects of overexpression of either full-length Hip (wild type (*wt*), *triangles*) or mutant Hip cDNA (*closed squares,* Hip-(1–219); *diamonds,* Hip-(15–368); *open squares,* Hip-(110–368)) on luciferase inactivation during ATP depletion (*circles,* vector only).

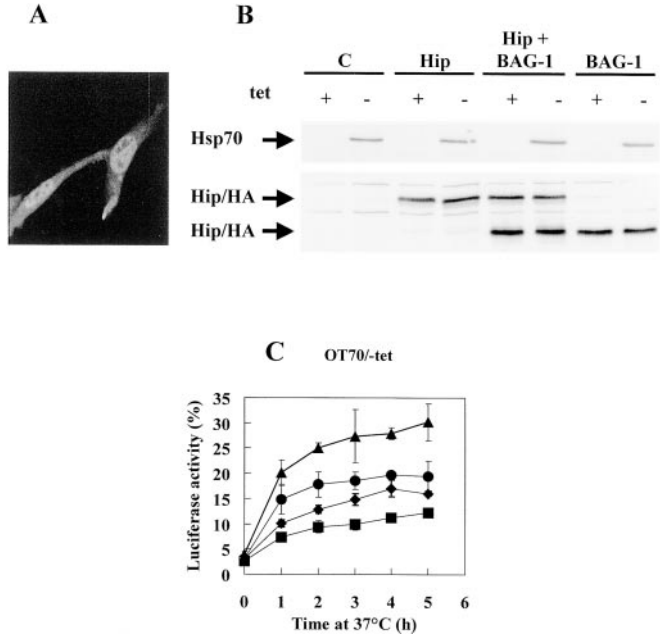

FIG. 4. **Dominant inhibition of the Hip-mediated enhancement of the Hsp70 chaperone activity by Bag-1.** OT70 cells were transiently transfected with pCytluc (encoding cytoplasmic luciferase) together with pCDNA (vector) or pCDNA/Hip, pCDNA/Bag-1 or pCDNA/Hip, and pCDNA/Bag-1 together. The cells were grown in medium with or without tetracycline (*tet*) for induction of Hsp70 expression. *A,* immunofluorescent analysis of HA-Bag-1 localization after staining the transfected cells with a monoclonal HA antibody. *B,* Western blot analysis of Hsp70, Hip, and Bag-1 expression. *C,* cells transfected with pCDNA (vector, *circles*), pCDNA/Hip (*triangles*), pCDNA/Bag-1 (*squares*), or pCDNA/Hip and pCDNA/Bag-1 (*diamonds*) were grown in medium without tetracycline (*tet*) for induction of Hsp70 expression. After pretreatment with cycloheximide (20 μg/ml), the cells were heated for 30 min at 45 °C to inactivate luciferase and allowed a recovery period of 0–5 h at 37 °C. Samples for luciferase activity were taken at the indicated time points. Data points represent the mean of 3 independent experiments, and *error bars* indicate S.E. of the means.

Our data on effects of chaperones on ATP depletion-induced protein damage revealed several new features. It seems plausible to assume that most if not all Hsp70s will be in the ADP-bound state under conditions of ATP depletion. This notion is supported by the fact that we could not detect a stimulating effect of Hsp40/Hdj-1 on the chaperone activity of Hsp70 in ATP-depleted cells. Hence, our data indicate that the ADP-bound Hsp70 can still act as a chaperone despite its lower substrate on rate (34). The data also imply that Hsp70 does not need to undergo cycling between the ATP and ADP states to protect against protein inactivation. This is consistent with the observation that mutant Hsp70s that lack their entire ATPase domain are still capable of protecting against proteotoxic damage (35). Finally, the finding that Hip is capable of stimulating the chaperone activity of Hsp70 under ATP-depleted conditions is consistent with the suggestion that Hip stabilizes the interaction of substrate with Hsp70 in its ADP-bound state. Indeed *in vitro* data showed that Hsc70 only binds to Hip immobilized on Ni^{2+}-nitrilotriacetic acid-agarose columns when preincubated with ADP but not ATP (8). Also, Hip cannot associate with the Hsc70 mutant K71E, a mutant that cannot hydrolyze ATP (11, 28). For the *in vivo* situation, these data imply Hsp70 and Hip may be functional to combat the protein damage as it occurs during ischemic insults where cellular ATP levels severely drop.

As both Hip and BAG-1 interact with the Hsp70 ATPase domain, we finally examined whether and how they may functionally compete in regulating the *in vivo* chaperone activity of Hsp70. *In vitro* data had already shown that Bag-1 binding to Hsp70 results in a displacement of the Hsp70 cofactors Hsp40, Hop, and Hip (15, 16, 36, 37) indicating that it acts dominantly over these other regulators. Indeed, we already demonstrated previously that physiological increases in the cellular level of Bag-1 inhibit the Hsp70-dependent chaperone activity in cells that constitutively express Hsp40 and Hip. This activity was seen for multiple isoforms of Bag-1 with molecular masses of 29, 33, 46, and 50 kDa that are expressed in cells as originating from different start codons (17). Here we used the 29-kDa isoform that is predominantly located in the nucleus but showed cytoplasmic localization and was capable of inhibiting the refolding of the heat-inactivated cytoplasmic luciferase (17). By using HA-tagged proteins, we could transfect transiently with Bag-1 and Hip such that equal total levels of the proteins were expressed. With Hip being exclusively cytoplasmic, we therefore concluded that it was present in the cytoplasm in excess over Bag-1. Still, under these conditions we

observed that Bag-1 could strongly diminish the refolding of heat-denatured cytoplasmic luciferase in the presence of excess Hip. So *in vivo*, like *in vitro*, Hsp70 prefers association with Bag-1 in the presence of Hip, and we conclude that the effects of positive and negative regulators of Hsp70 can be reproduced in both systems yielding the same results.

To what extent can we draw conclusions on the regulation of Hsp70 activity in dealing with stress-induced protein damage by overexpression of co-chaperones? Neither Hip nor BAG-1 are classical heat shock proteins as their levels are not induced in stressed cells, whereas Hsp70 and Hdj-1/Hsp40 are strongly induced and accumulate to high levels following heat shock or ATP depletion. Furthermore, the relative ratio of Hsp70 and co-chaperones changes within the cell due to the heat shock-induced translocation of Hsp70 and Hsp40 to the nucleus (29–32), whereas the localization of Hip and Bag-1 remains unchanged (Ref. 7 and data not shown). We previously showed that in hamster lung fibroblasts (O23 cells) elevated Hsp70 levels alone seemed to be sufficient to provide resistance toward cytoplasmic proteotoxicity comparable to that observed in pre-stressed cells. For the nuclear chaperone activity other factors seem to be required in addition to an increase in the level of Hsp70 to provide resistance comparable to that observed in pre-stressed O23 cells (21). However, under normal conditions of cell growth and differentiation, the levels of Hip and Bag-1 vary among different tissue culture cell lines and tissues (38). This suggests that the ultimate Hsp70 chaperone function both before and after stress could vary not only according to the total expression levels of Hsp70 but also to the levels of expression of the positive or negative co-chaperones, their affinity toward Hsp70, their cell-organelle-specific localization, and condition-mediated (re)distributions of the (co)chaperones.

Acknowledgment—We thank Prof. Dr. R. I. Morimoto (Evanston, IL) for helpful discussions.

REFERENCES

1. Hartl, F. U. (1996) *Nature* **381,** 571–580
2. Bukau, B., and Horwich, A. L. (1998) *Cell* **92,** 351–366
3. Kampinga, H. H. (1993) *J. Cell Sci.* **104,** 11–17
4. Freeman, B. C., Myers, M. P., Schumacher, R., and Morimoto, R. I. (1995) *EMBO J.* **14,** 2281–2292
5. Minami, Y., Hohfeld, J., Ohtsuka, K., and Hartl, F. U. (1996) *J. Biol. Chem.* **271,** 19617–19624
6. Michels, A. A., Kanon, B., Konings, A. W., Ohtsuka, K., Bensaude, O., and Kampinga, H. H. (1997) *J. Biol. Chem.* **272,** 33283–33289
7. Michels, A. A., Kanon, B., Bensaude, O., and Kampinga, H. H. (1999) *J. Biol. Chem.* **274,** 36757–36763
8. Hohfeld, J., Minami, Y., and Hartl, F. U. (1995) *Cell* **83,** 589–598
9. Irmer, H., and Hohfeld, J. (1997) *J. Biol. Chem.* **272,** 2230–2235
10. Bruce, B. D., and Churchich, J. (1997) *Eur. J Biochem.* **245,** 738–744
11. Prapapanich, V., Chen, S., Nair, S. C., Rimerman, R. A., and Smith, D. F. (1996) *Mol. Endocrinol.* **10,** 420–431
12. Ballinger, C. A., Connell, P., Wu, Y., Hu, Z., Thompson, L. J., Yin, L. Y., and Patterson, C. (1999) *Mol. Cell. Biol.* **19,** 4535–4545
13. Takayama, S., Sato, T., Krajewski, S., Kochel, K., Irie, S., Millan, J. A., and Reed, J. C. (1995) *Cell* **80,** 279–284
14. Takayama, S., Bimston, D. N., Matsuzawa, S., Freeman, B. C., Aimesempe, C., Xie, Z. H., Morimoto, R. I., and Reed, J. C. (1997) *EMBO J.* **16,** 4887–4896
15. Zeiner, M., Gebauer, M., and Gehring, U. (1997) *EMBO J.* **16,** 5483–5490
16. Hohfeld, J., and Jentsch, S. (1997) *EMBO J.* **16,** 6209–6216
17. Nollen, E. A., Brunsting, J. F., Song, J., Kampinga, H. H., and Morimoto, R. I. (2000) *Mol. Cell. Biol.* **20,** 1083–1088
18. Takayama, S., Xie, Z., and Reed, J. C. (1999) *J. Biol. Chem.* **274,** 781–786
19. Ohtsuka, K. (1993) *Biochem. Biophys. Res. Commun.* **197,** 235–240
20. Hunt, C., and Morimoto, R. I. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 6455–6459
21. Nollen, E. A., Brunsting, J. F., Roelofsen, H., Weber, L. A., and Kampinga, H. H. (1999) *Mol. Cell. Biol.* **19,** 2069–2079
22. Michels, A. A., Nguyen, V. T., Konings, A. W., Kampinga, H. H., and Bensaude, O. (1995) *Eur. J Biochem.* **234,** 382–389
23. Loktionova, S. A., Ilyinskaya, O. P., and Kabakov, A. E. (1998) *Am. J. Physiol.* **275,** H2147–H2158
24. Nguyen, V. T., and Bensaude, O. (1994) *Eur. J. Biochem.* **220,** 239–246
25. Kabakov, A. E., and Gabai, V. L. (1997) *Heat Shock Proteins and Cytoprotection: ATP-deprived Mammalian Cells*, pp. 49–77 Landes Co., Austin, TX
26. Kabakov, A. E., and Gabai, V. L. (1993) *Experientia* (*Basel*) **49,** 706–710
27. Luders, J., Demand, J., Schonfelder, S., Frien, M., Zimmermann, R., and Hohfeld, J. (1998) *Biol. Chem.* **379,** 1217–1226
28. Prapapanich, V., Chen, S. Y., and Smith, D. F. (1998) *Mol. Cell. Biol.* **18,** 944–952
29. Ohtsuka, K., Nakamura, H., and Sato, C. (1986) *Int. J. Hyperthermia* **2,** 267–275
30. Welch, W. J., and Mizzen, L. A. (1988) *J. Cell Biol.* **106,** 1117–1130
31. Pelham, H. R. B. (1984) *EMBO J.* **3,** 3095–3100
32. Hattori, H., Kaneda, T., Lokeshwar, B., Laszlo, A., and Ohtsuka, K. (1993) *J. Cell Sci.* **104,** 629–638
33. Boorstein, W. R., Ziegelhoffer, T., and Craig, E. A. (1994) *J. Mol. Evol.* **38,** 1–17
34. Beissinger, M., and Buchner, J. (1998) *Biol. Chem.* **379,** 245–259
35. Stege, G. J., Li, L., Kampinga, H. H., Konings, A. W., and Li, G. C. (1994) *Exp. Cell Res.* **214,** 279–284
36. Gebauer, M., Zeiner, M., and Gehring, U. (1998) *Mol. Cell. Biol.* **18,** 6238–6244
37. Gebauer, M., Zeiner, M., and Gehring, U. (1997) *FEBS Lett.* **417,** 109–113
38. Takayama, S., Krajewski, S., Krajewska, M., Kitada, S., Zapata, J. M., Kochel, K., Knee, D., Scudiero, D., Tudor, G., Miller, G. J., Miyashita, T., Yamada, M., and Reed, J. C. (1998) *Cancer Res.* **58,** 3116–3131