Heat shock protein (Hsp) 70 and Hsp40 expressed in mammalian cells had been previously shown to cooperate in accelerating the reactivation of heat-denatured firefly luciferase (Michels, A. A., Kanon, B., Konings, A. W. T., Ohtsuka, K., Bensaude, O., and Kampinga, H. H. (1997) J. Biol. Chem. 272, 33283–33289). We now provide further evidence for a functional interaction between Hsp70 and the J-domain of Hsp40 with denatured luciferase resulting in reactivation of heat-denatured luciferase within living mammalian cells. The stimulating effect of Hsp40 on the Hsp70-mediated refolding is lost when the proteins cannot interact as accomplished by their expression in different intracellular compartments. Likewise, the cooperation between Hsp40 and Hsp70 is lost by introduction of a point mutation in the conserved HPD motif of the Hsp40 J-domain or by deletion of the four C-terminal amino acids of Hsp70 (EEVD motif). Most strikingly, co-expression of a truncated protein restricted to the J-domain of Hsp40 had a dominant negative effect on Hsp70-facilitated luciferase reactivation. Taken together, these experiments indicate for the first time that the Hsp70/Hsp40 chaperones functionally interact with a heat-denatured protein within mammalian cells. The dominant negative effect of the Hsp40 J-domain on the activity of Hsp70 demonstrates the importance of J-domain-containing proteins in Hsp70-dependent processes.

In cells challenged by heat shock, several proteins unfold and aggregate. This damage is partially reversible as heat-denatured proteins can refold properly in cells allowed to recover from stress (1–3). During recovery from stress cells synthesize a group of stress inducible proteins, the so called heat shock proteins. Heat shock proteins (HSPs) are involved in degradation and reactivation of damaged proteins (reviewed in Refs. 4 and 5). Overexpression of one of these heat shock proteins, Hsp70, is sufficient to attenuate the heat-induced protein damage and accelerate its recovery in mammalian cells (6–9). Hsp70, a major stress-inducible chaperone, and its constitutively expressed cognate Hsc70 function in many cellular processes involving protein folding or unfolding (reviewed in Ref. 10). The protein refolding capacity of the eukaryotic Hsp70 s is influenced by several positive and negative modulator proteins (11–17). Positive modulators include the Hsp40 co-chaperone family, which enhances the ATPase activity of Hsp70 (18, 19), and Hip, which stabilizes the ADP-bound Hsp70 conformation (20). Bag-1 negatively modulates Hsp70 activity (13, 14, 21–23). Yet another cofactor, Hop, might function as a link between Hsp70 and Hsp90 (12). The present study is aimed at characterizing the Hsp70/Hsp40 cooperation within mammalian cells using reactivation of heat-inactivated firefly luciferase as an end point.

Hsp40 shares three evolutionary conserved domains with DnaJ (reviewed in Ref. 15). The N-terminal J-domain is separated from the C-terminal part by a glycine/phenylalanine-rich domain (the G/F-rich domain). The N-terminal J-domain that spans over the first 75 N-terminal amino acids is the most conserved between different species. It is also found in several proteins with no other homologies with the prokaryotic DnaJ or DnaK (27) and the adjacent G/F-rich domain is required. However, prokaryotic and eukaryotic mutants carrying only these two domains have reduced efficiencies compared with the wild type proteins. This suggests that parts of the C terminus are also involved in stimulating Hsp70 ATPase activity.

Firefly luciferase is a versatile reporter enzyme commonly used as a model to investigate protein refolding in vitro and protein damage and recovery in vivo. In vitro, refolding of chemically or thermally denatured luciferase is enhanced in the presence of Hsp70 or Hsc70 and either Hsp40 (18, 19) or the related Hdj2 protein (31). In mammalian cells, overexpression of the sole heat shock protein Hsp70 is sufficient to attenuate luciferase inactivation and to increase its reactivation (8). Furthermore, co-transfection of Hsp40 and Hsp70 results in an enhancement of the recovery of luciferase compared with recovery in the presence of Hsp70 alone (8). As Hsp40 and Hsp70 have many functions in the cellular metabolism, one cannot exclude that indirect effects rather than direct interactions between the chaperones and the heat-denatured luciferase are involved in the in vivo renaturation. To get further insight into
the cooperation between Hsp70 and Hsp40 at the molecular level in vivo, several mutants of the two chaperones were selected that are known to interfere with Hsp70/Hsp40 interaction or cooperation in vitro. The mutants were co-expressed in hamster O23 cells with luciferase, and their influence on reactivation of this reporter enzyme after heat-shock was investigated.

In the current report, we provide further evidence that a functional interaction between a J-domain protein and Hsp70 is required for Hsp70 chaperone activity within living mammalian cells. The stimulating effect of Hsp40 on Hsp70-mediated refolding is lost when the proteins cannot interact as accomplished by their expression in different intracellular compartments or by expression of mutant versions of both proteins incapable of physical interaction. Most strikingly, co-expression of a truncated protein restricted to the J-domain of Hsp40 full-length Hsp70 proteins completely inhibited the reactivation process. The latter finding suggests that J-domain proteins can strongly influence the Hsp70 function in a living cell and that mutation of these proteins might have severe consequences.

EXPERIMENTAL PROCEDURES

Plasmids and Cloning Techniques—Plasmid pRSVLL/LV (32), encoding a cytoplasmic localized firefly luciferase, was kindly provided by Dr. S. Subramani (University of California, San Diego, CA). To direct luciferase into the nucleus, we previously described the construction of pRSVnlsLV (33).

Plasmids pCMV40 and pCMV70 have been described previously (8). They were used to express the human Hsp40 (34) or the inducible human Hsp70 (35). The respective cDNAs were cloned in the eukaryotic expression vector pCMV5 (kindly donated by Dr. M. Stinsky, University of Iowa, Iowa City, IA).

Plasmid pCMV40 was used for the construction of Hsp40 mutants. Mutants were obtained via a polymerase chain reaction (PCR) using the appropriate primers and Tfi DNA polymerase (Promega). Plasmid pCMVnls40, encoding nuc-Hsp40, was constructed with the oligonucleotide 5'-TGA CGT CGT GCC GGT TAA AGA AGA CTA CTA CCA GAC GTT-3' (Eurosequence, Groningen, The Netherlands). The sense primer destroyed the Hsp40 start codon and introduced a new XhoI site, creating a truncated protein restricted to the J-domain of Hsp40 full-length Hsp70 proteins completely inhibited the reactivation process. The latter finding suggests that J-domain proteins can strongly influence the Hsp70 function in a living cell and that mutation of these proteins might have severe consequences.

Western Blotting—O23 cells in a 25-cm² dish were transfected with 1 μg of plasmid pCMV40, pCMVnls40, pCMV40H32Q, pCMV40–1–75, pCMV40–1–75H32Q, pCMV40–1–124, pCMV40–1–124H32Q, pCMV70, or pCMV70H4EVD or pSP64 as a control. In transient transfection assays, 10% of the O23 cell population is in the G1 phase and therefore the sensitivity of the recombinant protein detection, the O23 cells were co-transfected with 2 μg of plasmid pEGFP-N2, a vector encoding green fluorescent protein (GFP). The plasmid quantity was set at 10 μg/25-cm² dish by the addition of plasmid pSP64 (Promega). The green color enables to sort out cells expressing the GFP that express the various (mutant) proteins. The selected cell lysates were investigated by Western blot for expression of the various proteins. The cells were trypsinized 2 days after transfection. GFP-expressing cells were sorted on a Becton Dickinson flow cytometer. After sorting, SDS (1% (w/v)) and 2-mercaptoethanol were added to the cells, and they were sonicated and subsequently heated for 5 min at 90 °C. After electrophoresis through a 12.5% SDS-polyacrylamide gel, the proteins were electrotransferred onto nitrocellulose (Schleicher & Schüll).

Wild type Hsp40 and Hsp40 mutants were detected with an anti-Hsp40 antiserum (1:5,000 dilution) (SPA-400, Stress-gen), and Hsp70 and Hsp70H4EVD were detected with a monoclonal antibody directed against the inducible Hsp70 (1:2,000 dilution) (SPA-810, Stress-gen). Luciferase was detected with an anti-luciferase antibody (1:1,000) (Cor text). This was followed by the binding of a 1:5,000 diluted peroxidase conjugated donkey anti-rabbit antibody (Amersham Pharmacia Bio tech) for Hsp40 and 1:2,000 for luciferase, and a peroxidase-conjugated sheep anti-mouse antibody (Amersham Pharmacia Biotech) 1:2,000 diluted for Hsp70. Proteins were detected by enhanced chemiluminescence (ECL, Pierce).

Immunohistochemistry—One day after transfection, cells were plated on coverslips. One day after plating, the cells were processed. Immunofluorescence was performed at room temperature. The cells were incubated with PBS, fixed with 3% (v/v) formaldehyde for 15 min, then washed sequentially (three 5-min washes) with PBS and incubated 15 min with 0.2% (v/v) Triton X-100 in PBS, then 10 min with 100 mM glycine in PBS. Afterward, the coverslips were soaked for 30 min in 3% (v/v) bovine serum albumin in PBS and the cells were incubated in 1 h with a 1:150 dilution of an anti-Hsp40 serum in 0.1% (v/v) Tween 20 in PBS (TWEEN/PBS). The coverslips were washed in TWEEN/PBS and incubated for 1 h with a 1:200 dilution of a fluorescein conjugated anti-rabbit antibody (Nordic). After three washes in TWEEN/PBS, the coverslips were mounted with Vectashield (Vector Laboratories Inc.).
RESULTS

Hsp40 and Hsp70 Functionally Interact in Refolding Luciferase—Previously, we have shown that overexpression of Hsp70 is sufficient to attenuate luciferase inactivation and to increase its reactivation in mammalian cells. Co-expression of Hsp40 enhanced the Hsp70-facilitated luciferase recovery (8). This Hsp70-facilitated luciferase reactivation, however, might have been an indirect effect as the Hsp70/Hsp40 machinery has multiple targets in cells. Preventing or repairing a damage at one place might prevent or accelerate another damage in a different place through a series of events connected in cascade. To address this, we developed a strategy that had been successful in showing the in vivo interaction between the steroid receptor and Hsp90 (38). Luciferase can be targeted to either the cytoplasm or the nucleus without affecting its activity and thermal resistance in vitro (33, 39). wt-Hsp40 and Hsp70 expressed in O23 cells are localized both in the nucleus and the cytoplasm and cooperate in the reactivation of luciferase in both compartments (8). First, we directed Hsp40 and luciferase to the same intracellular compartment. A nuclear targeted Hsp40 (nuc-Hsp40) was engineered by fusing the SV40 large T antigen nuclear localization signal to the N terminus of Hsp40. The resulting nuc-Hsp40 expressed in O23 cells migrated slightly slower than wt-Hsp40, consistent with a higher molecular mass attributed to the fused sequence (Fig. 1A). Expression of either wt- or nuc-Hsp40 did not induce a general stress response, as evidenced by measurement of the level of the stress inducible Hsp110 (data not shown). Indirect immunofluorescence shows that the nuc-Hsp40 was exclusively present in the nucleus, whereas wt-Hsp40 was present in both the cytoplasm and the nucleus (Fig. 1B). Previous data (8) showed that transiently transfected wt-Hsp70 also localized in both cytoplasm and nucleus.

Next, recovery of luciferase activity after heat shock was investigated in O23 cells expressing Hsp70 with combinations of nuc- or cyt-luciferase and wt-Hsp40 or nuc-Hsp40. As described previously, the Hsp70-mediated reactivation of both luciferases was enhanced when a combination of the human Hsp70 and wt-Hsp40 was expressed (8). Only the reactivation of nuc-luciferase was enhanced in the presence of a combination of Hsp70 and nuc-Hsp40 (Fig. 2, A and B). The reactivation of cyt-luciferase was only enhanced in presence of Hsp70 and wt-Hsp40. Thus, Hsp40 is most efficient in facilitating recovery of luciferase when both luciferase and Hsp40 are present in the same cellular compartment.

To test further whether a direct association of Hsp40 and Hsp70 is required for the chaperoning activity on luciferase in a living cell, a Hsp40 mutant was constructed in which the conserved histidine was mutated into glutamine (H32Q mutant). In vitro data have shown that mutations in the HPD motif in the J-domain of Hsp40 proteins abolish the interaction with Hsp70 (reviewed in Ref. 40). This mutant Hsp40 was indeed not able to stimulate luciferase refolding in presence of Hsp70 (Fig. 4A). Taken together these results strongly suggest that a direct functional interaction between Hsp40, Hsp70, and luciferase is involved in the Hsp70-assisted luciferase reactivation.

Hsp70 can bind to denatured proteins and as such protect them from irreversible aggregation in vitro, but the literature is inconsistent on the requirements for J-domain proteins to perform this function (19, 41). Overexpression of just Hsp70 in mammalian cells retards the formation of nuclear protein aggregates (7). Consistent with these data, we found that expression of Hsp70 attenuated the heat-induced Triton X-100 insolubilization of luciferase (Table 1). Co-expression of Hsp40 with Hsp70, however, did not lead to an additional increase in the Hsp70-mediated protection against protein insolubilization.

Dominant-negative Effects of Truncated Hsp40 Proteins on the Hsp70-mediated Refolding of Luciferase—From in vitro data, it is known that Hsp40 enhances the ATPase activity of Hsp70 that is essential for Hsp70-facilitated protein folding (18, 19). The Hsp70 ATPase activity is stimulated with higher efficiency by the wild type protein than by a truncated protein restricted to the N-terminal J and G/F-rich domain of Hsp40. A truncated Hsp40 protein restricted to only the J-domain is inactive in stimulating the Hsp70 ATPase activity (19). To evaluate the importance of Hsp70 ATPase stimulation in the in vivo refolding process, the corresponding truncated Hsp40...

![Fig. 1. Localization of wt-Hsp40 and nuc-Hsp40. Panel A, cells were transiently transfected with plasmids encoding wt-Hsp40 or nuc-Hsp40 and lysates were loaded and the nitrocellulose was probed with anti-Hsp40. As control lysates, equal numbers of cells transfected with the empty expression vector pSP64 (control) were loaded. Panel B, O23 cells were transiently transfected with plasmids encoding wt-Hsp40 or nuc-Hsp40 and processed for indirect immunofluorescence with an anti-Hsp40 antisera. Nucleoli are shown by small arrows and the cytoplasm by large arrows.](image-url)
FIG. 2. Effect of nuclear localized Hsp40 on the recovery of nuc- and cyt-luciferase in presence of Hsp70. O23 cells were transiently transfected with pRSV.LnlsLLV (nuc-luciferase, A) or pRSV.LLVLV (cyt-luciferase, B) alone (open circles) or together with plasmids encoding Hsp70 (filled circles), Hsp40 and Hsp70 (filled squares), or nuc-Hsp40 and Hsp70 (filled triangles). After a heat shock for 30 min at 44 °C (nuc-luciferase) or 45 °C (cyt-luciferase), these cells were allowed to recover at 37 °C for 0–60 min in presence of cycloheximide before lysis and subsequent luciferase measurements. Activities are shown as percentages of the activities before heat shock (100%). Data are the mean of 2–10 experiments ± 1 S.E.

TABLE I

Heat-induced insolubilization of luciferase

O23 cells transiently transfected with cyt-luciferase alone or combined with Hsp70, or with both Hsp70 and Hsp40, were heated for 30 min at 45 °C and lysed immediately thereafter. The cell lysates were fractionated in supernatants and pellets by centrifugation at 12,000 × g for 15 min. Laemmli sample buffer was added to the supernatant fractions, and samples were loaded on SDS-polyacrylamide gels and probed with an anti-luciferase antibody (Cortex). Luciferase levels in the supernatant fractions were related to the levels in non-heated control cells (100%) via a densitometrical analysis. Data are the mean of three experiments.

| Transfection                  | Triton T X-100 soluble luciferase (%) |
|-------------------------------|--------------------------------------|
| No HSP                       | Control 30 min at 45 °C S.E.         |
| Hsp70                         | 100                                   |
| Hsp70 + Hsp40                 | 100                                   |

CDNAS were constructed; Hsp40-G contained both the J and the G/F-rich domain (124 N-terminal amino acids), and Hsp40-J was restricted to the J-domain (75 N-terminal amino acids) (Fig. 3A). The expression of the recombinant proteins was assayed by Western blotting. The mutant Hsp40 proteins were successfully expressed in O23 cells and had the expected sizes (Fig. 3B). Expression of abnormal proteins may trigger a stress response that would lead to the synthesis of the endogenous hamster heat shock proteins. However, this was not true as expression of the Hsp40 truncated and full-length proteins did not induce the synthesis of the strongly inducible Hsp110 (42) (data not shown).

In contrast to wt-Hsp40, co-expression of Hsp40-G did not enhance but rather inhibited the reactivation of luciferase in presence of wt-Hsp70 (Fig. 4B). Strikingly, co-expression of Hsp40-J even completely abolished the Hsp70-facilitated recovery of heat-inactivated luciferase (Fig. 4C). The ratio of wt-Hsp40 or Hsp40-J versus Hsp70 was varied by transfecting the cells with different quantities of plasmids encoding the corresponding cDNAs. The stimulating effect of wt-Hsp40 on Hsp70-mediated recovery only became significant when the plasmids encoding the corresponding cDNAs were transfected at a ratio close to 1:1. The inhibiting effect of the J-domain already became significant upon transfection of very low levels of the plasmid encoding Hsp40-J (Fig. 5). Introduction of the point mutation in the Hsp40-G (Hsp40-G-H32Q) and Hsp40-J (Hsp40-J-H32Q) mutants abolished their inhibiting activities (Fig. 4, B and C). Together, these results strongly suggest that the inhibiting effects of the Hsp40 mutants are dominant negative and result from a direct interaction with Hsp70.

The last C-terminal amino acids (EEVD) of Hsp70 are important for the cooperation between Hsp40 and Hsp70 (18). Cooperation between a similar Hsp70 mutant (Hsp70ΔEEVD) and Hsp40 was tested in living cells. Expression of Hsp70ΔEEVD to the same level as Hsp70 (Fig. 6A) attenuated luciferase heat inactivation to the same extent as wild type Hsp70. Also, luciferase recovery after heat shock was enhanced compared with control cells when Hsp70ΔEEVD was expressed, although this effect was slightly reduced compared with wt-Hsp70. Consistent with in vitro data (18), Hsp40 could not enhance Hsp70ΔEEVD-mediated refolding, indicating that the last four amino acids of Hsp70 are required for Hsp40/Hsp70 cooperation. However, as for wild type Hsp70, co-expression of the Hsp40-J mutant also completely inhibited the
Hsp70 and Hsp40 (filled squares) or co-transfected with a plasmid encoding for Hsp70 alone (open squares), or with pRSVLL/V (cyt-luciferase) alone (open circles). Hsp70 function is interfered by the presence of Hsp40 J-domain with the Hsp70 function. 

Interference of the Hsp40 J-domain with the Hsp70 stimulation, the absence of this motif still allows functional activity (27, 28, 40, 43). These data together strongly argue for a direct interaction between the J-domain of Hsp40 and Hsp70.

Co-overexpression of Hsp70 and Hsp40 has been previously shown to accelerate the reactivation of heat-denatured firefly luciferase in mammalian cells (8). Here we show that the strongest cooperation between Hsp40 and Hsp70 is observed when both luciferase and full-length Hsp40 are targeted to the same cellular compartment. The cooperation between the two chaperones was abolished by introducing the H32Q point mutation in the J-domain of Hsp40. This histidine is essential for Hsp40-like proteins to bind Hsp70 and stimulate its ATPase activity (27, 28, 40, 43). These data together strongly argue for a direct interaction between the J-domain of Hsp40 and Hsp70 that is essential for chaperoning denatured proteins in mammalian cells.

Expression of the truncated Hsp40-J mutant completely inhibited the Hsp70-facilitated refolding of luciferase in hamster cells. Expression of the Hsp40-G mutant that contains the J-domain plus the G/F-rich domain partially inhibited luciferase refolding. Both dominant negative effects are abolished when the H32Q mutation is introduced in the highly conserved HPD motif of the J-domain, suggesting that the mutants must interact with Hsp70 to exert their inhibiting effect. By their interaction with Hsp70, the Hsp40 mutant proteins may compete with the endogenous hamster Hsp40 and other endogenous J-domain-containing proteins and as such inhibit Hsp70 functions. Such interference has been described between Hsp40 and presumably auxilin for the Hsc70-assisted uncoating of coated vesicles (44). Similarly, overexpression of the Hsp1 J-domain also interferes with SV40 large T antigen binding to Hsc70 and T antigen-mediated activation of E2F-responsive promoters (45). Since the Hsp40-J mutant also inhibits the function of the Hsp70-EEVD, an interaction with the J-domain may involve the N-terminal ATPase domain of Hsp70 rather than its C-terminal end. Consistently, a recent NMR study revealed that the prokaryotic DnaJ J-domain interacts with the ATPase domain of DnaK loaded with Mg.ADP (26). It remains possible that the C-terminal parts of Hsp40 interact with the C terminus of Hsp70 as suggested before (21, 46). Such a two-point interaction of Hsp40 with Hsp70 may link a conformational change in the ATPase site of Hsp70 to a conformational change of its substrate binding domain (18). The inhibition of Hsp70 functions by expression of just a J-domain implicates that maybe any J-domain-containing protein may compete with Hsp40 for Hsp70 and interfere with the Hsp70 chaperone function in a living cell.

How can binding of the Hsp40 mutant proteins result in inhibition of Hsp70-facilitated refolding? A first possibility is that binding of Hsp40 to unfolded proteins is essential for their delivery to Hsp70 and that this capacity is lost in the Hsp40 mutants. Hsp40-like proteins such as the bacterial DnaJ and yeast Ydj1 can bind to unfolded peptides through their C-terminal domains and may deliver them on Hsp70 (15, 43, 47, 48). However, such association has not been shown for Hsp40, 

**DISCUSSION**

Co-overexpression of Hsp70 and Hsp40 has been previously shown to accelerate the reactivation of heat-denatured firefly luciferase in mammalian cells (8). Here we show that the strongest cooperation between Hsp40 and Hsp70 is observed when both luciferase and full-length Hsp40 are targeted to the same cellular compartment. The cooperation between the two chaperones was abolished by introducing the H32Q point mutation in the J-domain of Hsp40. This histidine is essential for Hsp40-like proteins to bind Hsp70 and stimulate its ATPase activity (27, 28, 40, 43). These data together strongly argue for a direct interaction between the J-domain of Hsp40 and Hsp70.
Hsp70 on the thermal inactivation and post-heat reactivation of numbers of pre-heated, thermotolerant cells (20 min at 44 °C shown). Their overexpression did not provoke a general stress response (data not shown). The two proteins did not lead to induction of Hsp110, indicative that TT 37 °C were loaded (open squares coding for Hsp70 (EEVD) (19), or unfolded nascent proteins (49, 50). A stronger effect of deletion of the C-terminal EEVD sequence of Hsp70 on the thermal inactivation and post-heat reactivation of luciferase. Panel A, cells were transiently transfected with plasmids encoding wt-Hsp70 or Hsp70ΔEEVD. Lysates of transfected cells were loaded, and the nitrocellulose was probed with anti-Hsp70. As control lysates, cells transfected with the empty expression vector pSP64 (control) were loaded. As a positive control for Hsp70 expression, equal numbers of pre-heated, thermotolerant cells (20 min at 44 °C + 16 h at 37 °C) were loaded (TT). The levels of Hsp70ΔEEVD and wild type Hsp70 (wt-Hsp70) proteins were similar. Furthermore, expression of the two proteins did not lead to induction of Hsp110, indicative that their overexpression did not provoke a general stress response (data not shown). Panel B, O23 cells transiently transfected with just pRSVLL/V (cyt-luciferase) (open circles) or co-transfected with plasmids encoding for Hsp70 (open squares), or Hsp70ΔEEVD (open triangles), were heat-shocked for 0–20 min at 42 °C and luciferase activity was measured afterwards. Panel C, O23 cells transiently transfected with pRSVLL/V (cyt-luciferase) alone (open circles) or co-transfected with plasmids encoding for Hsp70 (open squares), Hsp70 and Hsp40 (filled squares), Hsp70ΔEEVD (open triangles), or Hsp70ΔEEVD and Hsp40 (filled triangles) were heat-shocked for 30 min at 45 °C and allowed to recover at 37 °C for 0–60 min in presence of cycloheximide before lysis and subsequent luciferase measurements. Activities are shown as percentages of the activities before heat shock (100%). Data are the mean of two to nine experiments ± 1 S.E.

although it might be too labile to be evidenced with either luciferase (19) or unfolded nascent proteins (49, 50). A stronger argument against this hypothesis is provided by our current data showing that the Hsp40-G mutant that also does not contain the putative C-terminal substrate binding domain is much less inhibitory than the Hsp40-J mutant. Our data favor the possibility that the extent of inhibition by the Hsp40 mutants relates to the extent of their impairment in stimulating the Hsp70 ATPase activity. In contrast to the full-length Hsp40 proteins, the J-domains of Hsp40 and Rdj1, the rat homologue of Hdj2, are inefficient in stimulating the Hsc70 ATPase activity (19, 30). The Hsp40-G mutant is intermediate in ATPase stimulation between the full-length wt-Hsp40 and the Hsp40-J protein. Thus, binding of Hsp40-like proteins lacking a functional C terminus to Hsp70 may prevent the endogenous Hsp40 or other J-domain-containing proteins to bind Hsp70. This might affect the ATPase activity, and/or the cooperation with other Hsp70-modulators and thus the chaperone activity of Hsp70. Alternatively, binding of Hsp40 mutants to Hsp70 might trap Hsp70 irreversibly in a conformation unable to release bound, denatured proteins. A similar mechanism has been described for the small murine Bag-1 protein that inhibits Hsp70 functions by forming stable ternary complexes with nonnative substrates that do not release (23). An irreversible conformation change might also affect the ability of other Hsp70 modulators to interact with or to affect Hsp70.

The literature is inconsistent on Hsp70’s requirement for Hsp40 to stabilize heat-denatured nonnative proteins. In vitro Hsc70 can prevent heat-induced luciferase aggregation, but Hsp40 enhances this capacity (19). In another in vitro study, no protection of heat denatured luciferase from aggregation could be observed by Hsc70 alone, and the presence of the J-domain protein cysteine string protein was required for this function (41). Our data imply that in a living cell Hsp40-mediated ATPase stimulation is not required for Hsp70-mediated protection during heat shock, or that the intrinsic or peptide stimulated ATPase level is already sufficient. First, Hsp40 co-expression did not enhance Hsp70-mediated protection against loss of luciferase enzyme activity during heat shock (8). Second, no effect of overexpressed Hsp40 or mutant Hsp40 proteins was seen on Hsp70-mediated protection against luciferase aggregation during heat shock. The latter is consistent with our previous data (51) showing that expression of a Hsp70 mutant completely lacking its ATPase domain did prevent protein aggregation during heat shock. Third, the expression of Hsp70ΔEEVD also protected luciferase from heat inactivation despite its increased ATPase activity that cannot be enhanced by Hsp40 (18). Apparently, no extra acceleration of the Hsp70 ATPase activity by Hsp40 is required for Hsp70 to be effective to bind and prevent heat-induced protein aggregation in living cells. Our in vivo data suggest that extra Hsp40 only seems required for efficient refolding of Hsp70-bound substrates.

We observed that expression of the Hsp70ΔEEVD mutant was almost as efficient as that of wt-Hsp70 in attenuating luciferase heat inactivation. Hsp70ΔEEVD could assist recovery, although less efficient than wt-Hsp70. Co-expression of wt-Hsp40 did not enhance its efficiency in vivo, in accordance with in vitro data (18) that Hsp40 cannot enhance luciferase refolding by this mutant Hsp70. However, in contrast to the in vivo situation, Hsp70ΔEEVD is completely inactive in protein refolding in vitro. Furthermore, if Hsp70 depends on Hsp40/ Hdj1 for refolding and Hsp70ΔEEVD cannot be affected by Hsp40/Hdj1, why did we observe enhanced refolding in presence of Hsp70ΔEEVD in vivo? Besides Hsp40, several other endogenous J-domain-containing proteins are present in the cell that can act as co-chaperones of Hsp70 and may enable Hsp70ΔEEVD activity as well. These include Hdj2, its rat homologue Rdj1, cysteine string protein, and auxilin (30, 31, 41, 52). At least auxilin and Rdj1 are reported to be able to modulate the chaperone activity of Hsp70 mutants with large C-terminal deletions including the EEVD sequence (30, 52). Consistently we found that the expression of the J-domain also abolished the chaperone activity of the Hsp70ΔEEVD mutant.
Alternatively, binding of the Hsp40 J-domain to Hsp70ΔEVD may block its intrinsic ATPase activity and/or trap the chaperone in a conformation that impairs cooperation with other modulators. This may lead to inhibitory effects on luciferase refolding.

Our findings that the interaction with functional J-domain-containing proteins strongly influences the chaperone activity of Hsp70 in a living cell, and that mutation of J-domain-containing proteins severely affects the Hsp70 chaperone activity, emphasize the important role of J-domain proteins in a living cell. Mutation of J-domain proteins might affect several functions of the Hsp70 chaperone, e.g. in both stress-induced thermotolerance and physiological functions, such as protein translation, protein transport, regulation of p53 and pRb, and activation of steroid receptors. Overexpression of mutant Hsp40 proteins now provides us with an excellent tool to knock out Hsp70 and see how this affects these processes. An impaired cooperation between J-domain proteins and Hsp70 is suggested to be involved in the development of rheumatoid arthritis (53). Furthermore, a recent report shows that the J-domain protein Hdj2 is essential for the prevention of arthritis (53). Furthermore, a recent report shows that the J-domain protein Hdj2 is essential for the prevention of arthritis (53).

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