J. Biochem. **135**, 193–200 (2004)
DOI: 10.1093/jb/mvh023

Modulation of Chaperone Activities of Hsp70 and Hsp70-2 by a Mammalian DnaJ/Hsp40 Homolog, DjA4

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Received October 8, 2003; accepted November 28, 2003

Type I DnaJs comprise one type of Hsp70 cochaperones. Previously, we showed that two type I DnaJ cochaperones, DjA1 (HSDJ/Hdj-2/Rdj-1/dj2) and DjA2 (cpr3/DNAJ3/Rdj-2/dj3), are important for mitochondrial protein import and luciferase refolding. Another type I DnaJ homolog, DjA4 (mmDjA4/dj4), is highly expressed in heart and testis, and the coexpression of Hsp70 and DjA4 protects against heat stress-induced cell death. Here, we have studied the chaperone functions of DjA4 by assaying the refolding of chemically or thermally denatured luciferase, suppression of luciferase aggregation, and the ATPase of Hsp70s, and compared these activities with those of DjA2. DjA4 stimulates the hydrolysis of ATP by Hsp70. DjA2, but not DjA4, together with Hsp70 caused denatured luciferase to refold efficiently. Together with Hsp70, both DjA2 and DjA4 are efficient in suppressing luciferase aggregation. bag-1 further stimulates ATP hydrolysis and protein refolding by Hsp70 plus DjA2 but not by Hsp70 plus DjA4. Hsp70-2, a testis-specific Hsp70 family member, behaves very similarly to Hsp70 in all these assays. Thus, Hsp70 and Hsp70-2 have similar activities *in vitro*, and DjA2 and DjA4 can function as partner cochaperones of Hsp70 and Hsp70-2. However, DjA4 is not functionally equivalent in modulating Hsp70s.

Key words: DnaJ homolog, Hsp70, Hsp70-2, protein aggregation, protein folding.

Abbreviations: Hsc70, 70 kDa heat shock cognate protein; Hsp70, 70 kDa heat shock protein; Hsp70-2, testis-specific 70 kDa heat shock protein.

Molecular chaperones of the 70 kDa heat shock protein family (Hsp70s) function in a diverse set of processes, including protein folding, multimer association and dissociation, translocation of proteins across membranes, and the regulation of heat shock response (*1*, *2*). The expressions of some members of the Hsp70 family are inducible by various stresses, whereas the expression of other members is constitutive. Some members are regulated developmentally during spermatogenesis (*3*). The best known mammalian cytosolic Hsp70s are Hsc70, which is expressed constitutively, and Hsp70, which is induced by stress conditions. In addition, two unique members, Hsp70-2 and Hsc70t, are expressed during spermatogenesis (*4*). The amino acid sequence of mouse Hsp70-2 is 86% and 83% identical to mouse Hsc70 and Hsp70, respectively. Hsc70, Hsp70, and Hsp70-2 are all highly conserved among mammals (about 99% identity between the human and mouse proteins). Hsp70-2 is not heat-inducible and exhibits stage-specific expression during spermatogenesis (*5*).

The chaperoning activity of Hsp70s is controlled by the ATPase domain, which undergoes a reaction cycle of ATP binding, hydrolysis, and nucleotide exchange (*6*). ATP hydrolysis converts Hsp70 from an open state exhibiting high association and dissociation rates for substrates, to a closed state exhibiting low association and dissociation rates (*7*). This cycle is regulated by cochaperones that stimulate the rate of ATP hydrolysis, such as members of the DnaJ family, and by cofactors that regulate nucleotide exchange, such as GrpE in the bacterial system (*8*, *9*). A number of DnaJ/Hsp40 family members and positive and negative regulators of nucleotide exchange have been identified in mammals. bag-1 has been reported to be a nucleotide exchange factor for Hsc70 and Hsp70 (*10*, *11*), but its precise role in the chaperone cycle is still unknown.

Mammalian cells have a large and diverse family of DnaJ proteins. All DnaJs have the Hsp70 binding domain (J domain) but differ in other domains, which allows DnaJ proteins to associate with distinct cellular substrates and to specifically target the partner Hsp70 of these substrates. To date, more than 40 Hsp40/DnaJ homologs have been identified in mammals and are classified into three groups based upon the degree of domain conservation with *E. coli* DnaJ (*12*). Type I DnaJ homologs possess all three domains of bacterial DnaJ, including the N-terminal, highly conserved J domain of about 70 amino acid residues, a glycine/phenylalanine-rich domain, and a cysteine-rich zinc-finger domain. Type II members contain the N-terminal J domain and the G/F-rich domain, but lack the zinc-finger domain. Type III members possess only the J domain. The J domain contains the hallmark His-Pro-Asp (HPD) motif, which is essential for interaction with Hsp70s (*13*). Some type I

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DnaJ proteins bind directly to unfolded protein substrates through their zinc-finger and C-terminal domains and may serve to target Hsp70s to substrates. This has been demonstrated for *E. coli* DnaJ (*14*), the yeast cytosolic DnaJ protein Ydj1p (*15*), and the yeast mitochondrial DnaJ protein Mdj1p (*16*).

In mammals, three type I DnaJ homologs have been identified in cytosol: DjA1 (HSDJ/Hdj-2/Rdj-1/dj2) (*17*, *18*), DjA2 (cpr3/DNAJ3/Rdj-2/dj3) (*19*), and DjA4 (mmDjA4/dj4) (*20*). On the other hand, DjB1 (hsp40/Hdj-1/dj1) (*21*, *22*) is a type II homolog. DjB1 has been reported to mediate the initial folding process of cytosolic proteins in cooperation with Hsc70 and the TRiC complex (*23*). We found that DjA1 and DjA2, but not DjB1, in combination with Hsc70, facilitate mitochondrial protein import and luciferase refolding (*24*, *25*). Recently we reported some characteristics of the newly-found DnaJ homolog, DjA4. DjA4 was found to be expressed strongly in mouse testis (*26*). Because Hsp70-2 is also expressed specifically in mouse testis (*27*), we speculated that DjA4 may be a partner chaperone of Hsp70-2.

In the present study, we analyzed the effects of DjA4 on luciferase refolding, heat-induced luciferase aggregation and ATPase activity by Hsp70s in comparison with those of DjA2. *In vitro* assay showed that purified recombinant DjA4 stimulates the ATPase activity of human Hsp70 and mouse Hsp70-2. DjA2 together with Hsp70 or Hsp70-2 was effective both in assisting luciferase refolding and in protecting against protein aggregation. In contrast, DjA4 was effective in protecting against protein aggregation, but not in assisting luciferase refolding. Different effects of bag-1 on Hsp70–DjA4 and Hsp70–DjA2 pairs are also described.

MATERIALS AND METHODS

Plasmid Construction—Mouse DjA4 cDNA (GenBank, accession number AB032401) was amplified by polymerase chain reaction (PCR) using mouse testis cDNA and cloned into the *Hinc*II site of pGEM-3Zf(+) (Promega, Madison, WI, USA) to generate pGEM-mDjA4. The PCR oligonucleotides were 5′-GAACGGAGACAAGATGGTGAAGG-3′ (sense) and 5′-CATTCATCATGTACTAGAGTCC-3′ (antisense), giving and yielded a fragment of 1,256 base pairs. A second PCR was done using 5′-ATGCATCACCATCACCATCACATGGTGAAGGAGACCCAGTACTATG-3′ (sense) and 5′-GTCATAGCTGTTTCCTG-3′ (antisense) as the primers and pGEM-mDjA4 as template to construct N-terminal hexahistidine-tagged mDjA4 (H_6mDjA4). The resultant DNA fragment was inserted into the blunt-ended *Sap*I site of the pTYB11 vector (New England Biolabs, Beverly, MA, USA) to generate pTYB-H_6mDjA4. The plasmid was digested with *Bam*HI and *Eco*RI and the resulting fragment containing the H_6m-DjA4 sequence was finally inserted into the *Bam*HI–*Eco*RI sites of pVL1393 baculovirus transfer vector (PharMingen, San Diego, CA, USA) yielding pVL-H_6m-DjA4.

Mouse Hsp70-2 cDNA (GenBank, accession number BC004714) was amplified by PCR from mouse testis cDNA using a pair of oligonucleotides, 5′-TCAGTCAGGATGTCTGCCCG-3′ (sense) and 5′-AGGTTTACGCGGACTCCAGC-3′ (antisense), giving a fragment of 1,937 base pairs. The PCR product was cloned into the *Sma*I site of pGEM-3Zf(+) and designated pGEM-mHsp70-2. The *Eco*RI–*Pst*I fragment containing the mHsp70-2 sequence was then excised from the pGEM-mHsp70-2 and subcloned into the *Pst*I–*Eco*RI sites of pVL1392 baculovirus transfer vector (PharMingen) yielding pVL-mHsp70-2.

The human inducible Hsp70 cDNA (*28*) was subcloned into the *Bam*HI–*Not*I sites of pVL1393 vector yielding pVL-hHsp70. The nucleotide sequences were verified by sequencing.

Expression and Purification of Chaperones—Mouse H_6DjA4, mouse Hsp70-2 and human Hsp70 were expressed using the insect-baculovirus system. Sf9 insect cells were infected with recombinant baculovirus transfer vector (pVL-H_6mDjA4, pVL-mHsp70-2, or pVL-hHsp70) with linear viral BaculoGold DNA (BaculoGold transfection kit; PharMingen), and high titer viral stocks were prepared according to the manufacturer's protocol.

The Sf9-expressed H_6DjA4 was purified by Ni^{2+}-NTA-Sepharose (Amersham Biosciences, Buckinghamshire, UK) column chromatography. Briefly, H_6DjA4-expressing Sf9 cells (2.2 g wet weight) were suspended in 20 ml of lysis buffer [buffer A (20 mM HEPES-KOH, pH 7.4, 0.75 M NaCl, 10% glycerol) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml chymostatin, 1 μg/ml antipain and 1 μg/ml leupeptin)] and lysed by sonication using a Sonifier Cell Disruptor (Branson Ultrasonics Corporation, Danbury, CT, USA). Cell lysates were centrifuged at 100,000 ×*g* for 40 min and the supernatants were applied to a Ni^{2+}-NTA-Sepharose column (1 ml). The column was washed with buffer A containing 60 mM imidazole, and then H_6DjA4 was eluted with buffer A containing 1 M imidazole. Finally, the eluate was dialyzed against buffer A and concentrated with Centricon YM-30 (Amicon, Beverly, MA, USA).

Hsp70-2- and Hsp70-expressing Sf9 cells (each about 7 g wet weight) were lysed by sonication in 50 ml of buffer B (20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.5 mM DTT) containing protease inhibitors. Cleared cell lysates were loaded onto 50 ml DEAE-Sepharose (Amersham Biosciences) columns equilibrated with buffer B. Bound proteins were eluted with a linear gradient of 0–500 mM KCl in buffer B. The peak fractions containing Hsp70-2 and Hsp70 were pooled and applied to 5 ml ATP-agarose (Sigma: C8-linkage) columns equilibrated with buffer C (20 mM HEPES-NaOH, pH 7.2, 0.1 M NaCl, 5 mM $MgCl_2$, 1 mM DTT) containing protease inhibitors. The columns were then washed with buffer C, and Hsp70-2 and Hsp70 were eluted with buffer C supplemented with 5 mM ATP. The eluted Hsp70-2 and Hsp70 were concentrated and finally applied to a Superdex 200 pg gel filtration column (120 ml, Amersham Biosciences) equilibrated with buffer C. The fractions containing homogenous Hsp70-2 and Hsp70 were collected, concentrated, and used as the purified chaperones.

Human H_6DjA2 was expressed in an Sf9-baculovirus expression system and purified as described (*25*). Mouse H_6bag-1 was expressed in *E. coli* and purified as described (*25*).

Refolding of Chemically-Denatured Luciferase—Firefly luciferase (Sigma) was dissolved to 5.0 μM in buffer D

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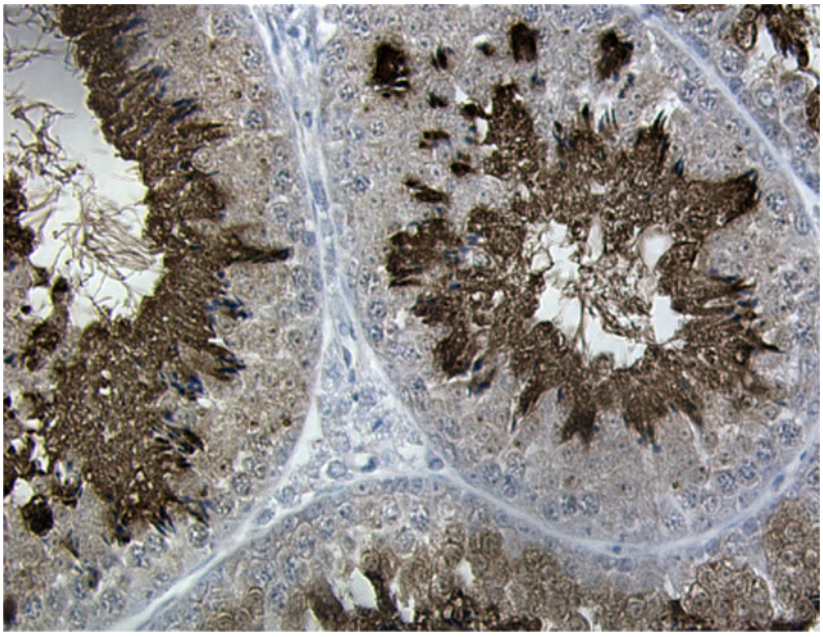

Fig. 1. **Immunohistochemical analysis of DjA4 in mouse testis.** Mouse (3 months old) testis was immersed in bouin fixative solution, embedded in paraffin, and 5 μm-sections were prepared. The deparaffinized section was boiled in 10 mM citrate buffer (pH 6.0) to increase the antigenicity. The section was incubated with antiserum against mouse DjA4 (1:1,000 dilution) as the primary antibody. Signals were amplified by a Tyramide Signal Amplification kit (PerkinElmer Life Sciences, Boston, MA, USA) according to the manufacturer's protocol. Peroxidase activity was visualized using 3,3′-diaminobenzidine as a substrate.

(20 mM HEPES-KOH, pH 7.4, 120 mM potassium acetate, 6 M guanidine hydrochloride) and denatured for 60 min at 25°C. The denatured luciferase was placed on ice and diluted 1:40 in buffer E (25 mM HEPES-KOH, pH 7.2, 50 mM potassium acetate, 5 mM DTT). Then 1.0 μl of diluted luciferase was added to 24 μl of refolding buffer (28 mM HEPES-KOH, pH 7.6, 120 mM potassium acetate, 1.2 mM magnesium acetate, 2.2 mM DTT, 1 mM ATP, 8.8 mM creatine phosphate, 7 units/ml creatine kinase, 16 μM BSA) with or without chaperone(s). Refolding was started by incubating the samples at 25°C. At the indicated times, 1.0 μl aliquots were withdrawn from the folding reaction mixture and the refolded activity was measured as described (*24*). Native luciferase activity was taken as 100%.

Refolding of Heat-Denatured Luciferase—Native luciferase (2.0 μM in 20 mM HEPES-KOH, pH 7.4, 120 mM potassium acetate) was diluted 1:25 into 25 μl of refolding buffer in the presence of various molecular chaperones. The reaction mixture was equilibrated at 25°C, incubated at 42°C for 10 min (inactivation period) and subsequently incubated at 25°C (recovery period). Aliquots of 1.0 μl were withdrawn at different time points, diluted in refolding buffer, and luciferase activity was measured. The luciferase activity was expressed as a percent of a non-heat denatured sample.

Luciferase Aggregation Assay—To monitor the thermal aggregation of luciferase, buffer F (28 mM HEPES-KOH, pH 7.6, 120 mM potassium acetate, 1.2 mM magnesium acetate, 2.2 mM DTT, 1 mM ATP, 8.8 mM creatine phosphate, 3.5 units/ml creatine kinase) containing chaperone proteins was prewarmed to 43°C in a stirred quartz cuvette. The cuvette was immediately transferred into a thermostated cell holder in an F-4500 fluorescence spectrophotometer (Hitachi, Tokyo). The aggregation assay was started by adding 100-fold diluted native luciferase (0.2 μM, final concentration) into the reaction mixture, and light scattering was monitored at 43°C. The excitation and emission wavelengths were both 320 nm, with spectral bandwidths of 2.5 nm. The measured light scattering was normalized by subtracting the background at zero time.

Other Methods—The ATPase assay was carried out as described previously (*25*). Protein concentration was determined with a protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as a standard. Western blotting was performed using a polyclonal antibody against human Hsp70 (Hsp70-specific, K-20, Santa Cruz, CA, USA) or antiserum against mouse DjA4 (*26*). Polypeptides were visualized using an enhanced chemiluminescence kit (Amersham Biosciences). The intensity of chemiluminescence was quantitated using an LAS1000plus image analyzer (Fuji Photo Film, Tokyo).

RESULTS

Immunohistochemical Analysis of DjA4 in Testis—Immunohistochemical analysis of DjA4 in the testis from adult mouse was performed (Fig. 1). Round and elongated spermatids were strongly positive for DjA4 protein, spermatocytes were weakly positive and Leydig cells were negative. The localization of DjA4 accords well with the expression pattern in fractionated testicular cells (data not shown).

Intracellular Concentrations of Chaperones—We purified recombinant human Hsp70, mouse Hsp70-2 and mouse H_6DjA4. Purified H_6DjA4 migrated as a polypeptide of 47 kDa in SDS–8% polyacrylamide gels (Fig. 2A). Purified Hsp70 and Hsp70-2 migrated as polypeptides of 70 kDa.

We then determined the intracellular concentrations of DjA4 and Hsp70 in HeLa cells by Western blotting, using purified recombinant mouse H_6DjA4 and human Hsp70 as standards. The Hsp70 concentration was estimated to be about 0.8 mg/g cells (12 μM of total protein) (Fig. 2B). The concentration of DjA4 was estimated to be 0.1 mg/g cells (2.5 μM of total protein). The slight difference in

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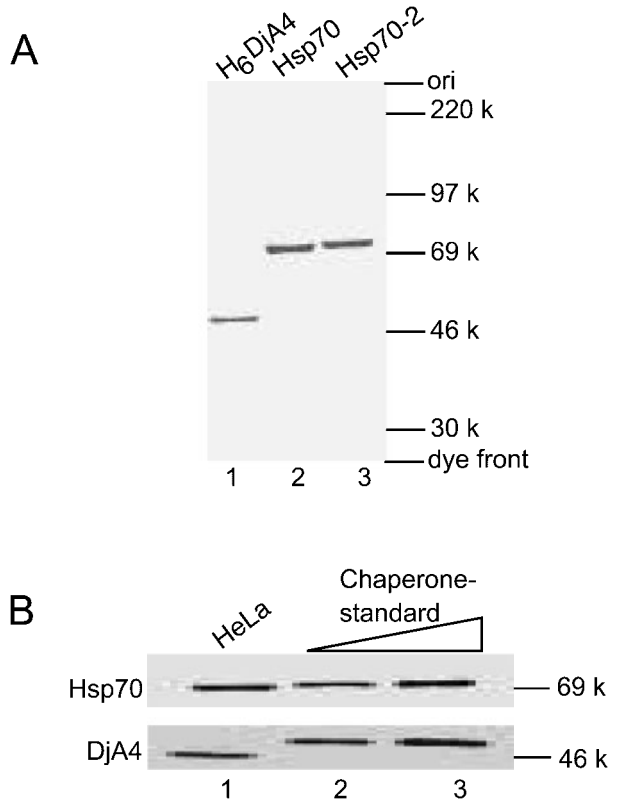

Fig. 2. **Intracellular concentrations of chaperones.** (A) Purified chaperones (0.5 μg each) were analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining. Lane 1, hexahistidine-tagged mouse DjA4; lane 2, human Hsp70; lane 3, mouse Hsp70-2. (B) Immunoblot analysis of Hsp70 (top) and DjA4 (bottom) in a HeLa total cell extract (5 μg protein for Hsp70 and 10 μg protein for DjA4). Purified recombinant chaperones were used as standards (lanes 2 and 3): top, human Hsp70 (10 ng and 20 ng); bottom, hexahistidine-tagged mouse DjA4 (5 ng and 10 ng). The positions of protein molecular mass markers (rainbow-colored markers; Amersham Biosciences) are indicated on the right.

migration of endogenous DjA4 from HeLa cells and the standard is due to the hexahistidine tag.

Refolding of Chemically-Denatured Luciferase by Purified Chaperones—Previously, we found that DjA1 and DjA2 in combination with Hsc70 facilitate luciferase refolding (*24*, *25*). We asked whether DjA4 also has luciferase refolding activity in combination with Hsp70 family proteins. We used purified chaperone proteins in the refolding assay. When chemically denatured luciferase was incubated with bovine serum albumin (negative control), a slight refolding of the enzyme was observed (Fig. 3A). Hsp70 (1.8 μM), Hsp70-2 (1.8 μM), DjA2 (0.4 μM), or DjA4 (0.4 μM) alone did not stimulate refolding.

When DjA2 together with Hsp70 or Hsp70-2 was added, luciferase refolding occurred (Fig. 3, B and C). Refolding was slow for the initial 10 min and proceeded thereafter. These results are in accord with the previous results with the Hsc70 plus DjA2 chaperone pair (*25*). When 0.4 μM bag-1 was additionally added, there was no initial lag and the refolding was much enhanced, and about 80% of luciferase activity was recovered in 60 min. Similar results were obtained with the chaperone system of Hsc70, DjA2 and bag-1 (*25*).

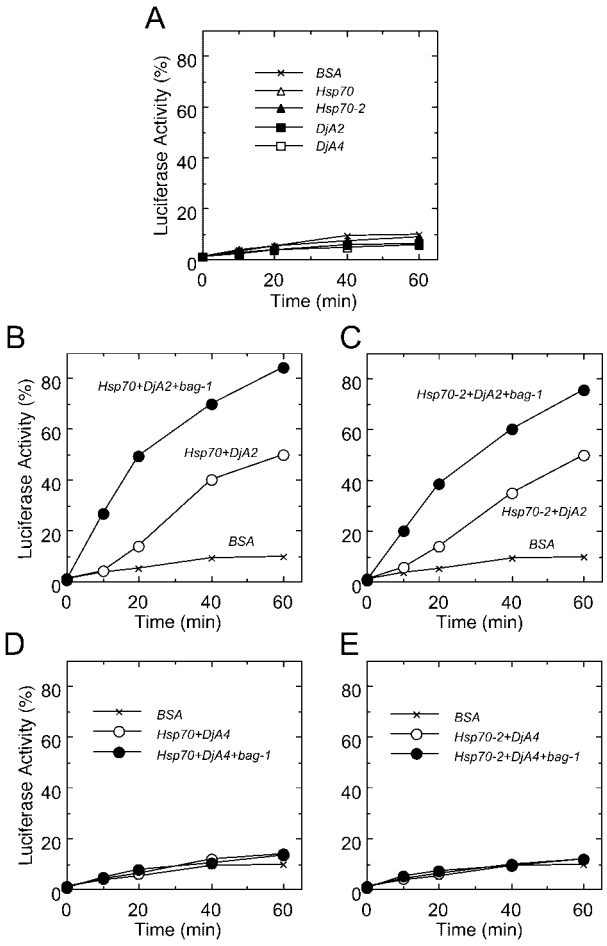

Fig. 3. **Refolding of chemically-denatured luciferase by purified chaperones.** Chemically-denatured luciferase (300 ng/ml, 5.0 nM) was refolded for 60 min at 25°C. The activity of firefly luciferase in the presence of individual chaperones or in combination was measured at the indicated times. (A) Effect of Hsp70, Hsp70-2, DjA2, and DjA4 on luciferase refolding. (B) Effect of Hsp70, DjA2 and bag-1 on luciferase refolding. (C) Effect of Hsp70-2, DjA2, and bag-1 on luciferase refolding. (D) Effect of Hsp70, DjA4 and bag-1 on luciferase refolding. (E) Effect of Hsp70-2, DjA4, and bag-1 on luciferase refolding. Bovine serum albumin (BSA) was included as a negative control. Final concentrations of chaperones were: Hsp70 and Hsp70-2, 1.8 μM; DjA2, DjA4, and bag-1, 0.4 μM.

The effect of DjA4 on luciferase refolding was monitored. When DjA4 together with Hsp70 or Hsp70-2 was added, luciferase refolding was not stimulated (Fig. 3, D and E). Further, the addition of bag-1 had no effect on refolding in the case of either chaperone pair. DjA4 was not effective in refolding in combination with Hsc70 and/or bag-1 (data not shown).

Refolding of Thermally-Denatured Luciferase by Chaperones—We next studied the refolding activity of chaperones using thermally-denatured luciferase. In the absence of chaperones, luciferase was readily unfolded by mild heating at 42°C and did not fold spontaneously upon lowering the temperature (see Fig. 4, A and B). The presence of Hsp70 or Hsp70-2, DjA2 or DjA4 and bag-1 in various combinations hardly protected luciferase during thermal denaturation as judged from the activity at zero time. Hsp70 or Hsp70-2 alone was not effective in assist-

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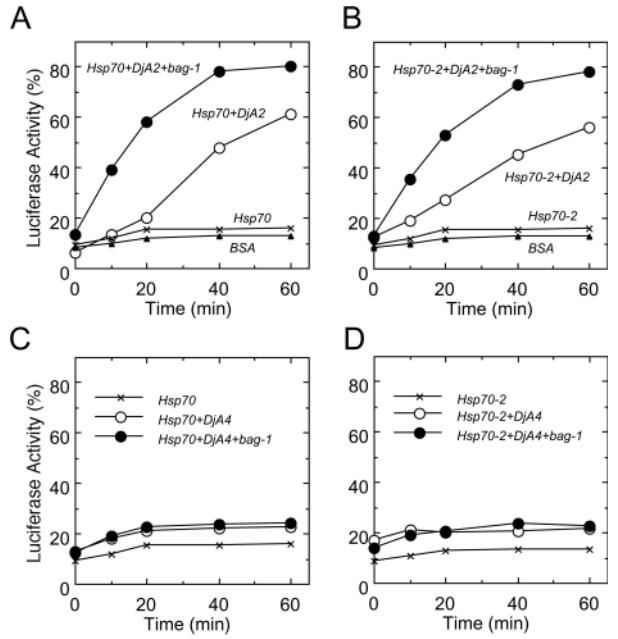

Fig. 4. **Refolding of thermally-denatured luciferase by purified chaperones.** Native luciferase (4.8 μg/ml, 80 nM) was equilibrated at 25°C in the presence of the indicated combinations of chaperones, denatured at 42°C for 10 min, and then allowed to refold by shifting the temperature back to 25°C. Luciferase activity was measured for 60 min at 25°C in the presence of the indicated chaperones. DjA2 and DjA4 alone had no apparent effect on luciferase refolding. The concentrations of the chaperones were the same as described in the legend to Fig. 3.

ing refolding. However, DjA2 in combination with Hsp70 or Hsp70-2 could promote luciferase refolding. bag-1 further stimulated the refolding in the cases of both chaperone pairs. The refolding kinetics of thermally denatured luciferase was very similar to that of chemically denatured luciferase (Figs. 3 and 4). In contrast, DjA4 in combination with Hsp70 or Hsp70-2 was not effective in assisting folding (Fig. 4, C and D). Increasing the amount of DjA4 (0.5–2.0 μM) in combination with either Hsp70 or Hsp70-2 had no effect (data not shown). Furthermore, the addition of bag-1 did not improve the refolding by the chaperone pairs of Hsp70 plus DjA4 or Hsp70-2 plus DjA4.

These results show that Hsp70 and Hsp70-2 (and also Hsc70), in combination with DjA2, are functionally similar in refolding activity of either chemically or thermally denatured luciferase. bag-1 accelerates refolding by these chaperone pairs. In contrast, DjA4 does not facilitate luciferase refolding in combination with Hsp70 or Hsp70-2 (or Hsc70) and bag-1.

Suppression of Luciferase Aggregation by Chaperones—Next, we examined the effects of purified chaperones on luciferase aggregation by light scattering. The effects of Hsp70 plus DjA2 or DjA4 are shown in Fig. 5 (A and B). When luciferase (0.2 μM) was incubated at 43°C in the absence of chaperone, it began to aggregate after 2 min, reached a maximum at around 10 min, and decreased gradually afterwards. This decrease in light scattering is presumably due to the formation of larger aggregates.

Aggregation was slightly inhibited in the presence of a 2.5-fold molar excess of Hsp70. In the presence of DjA2,

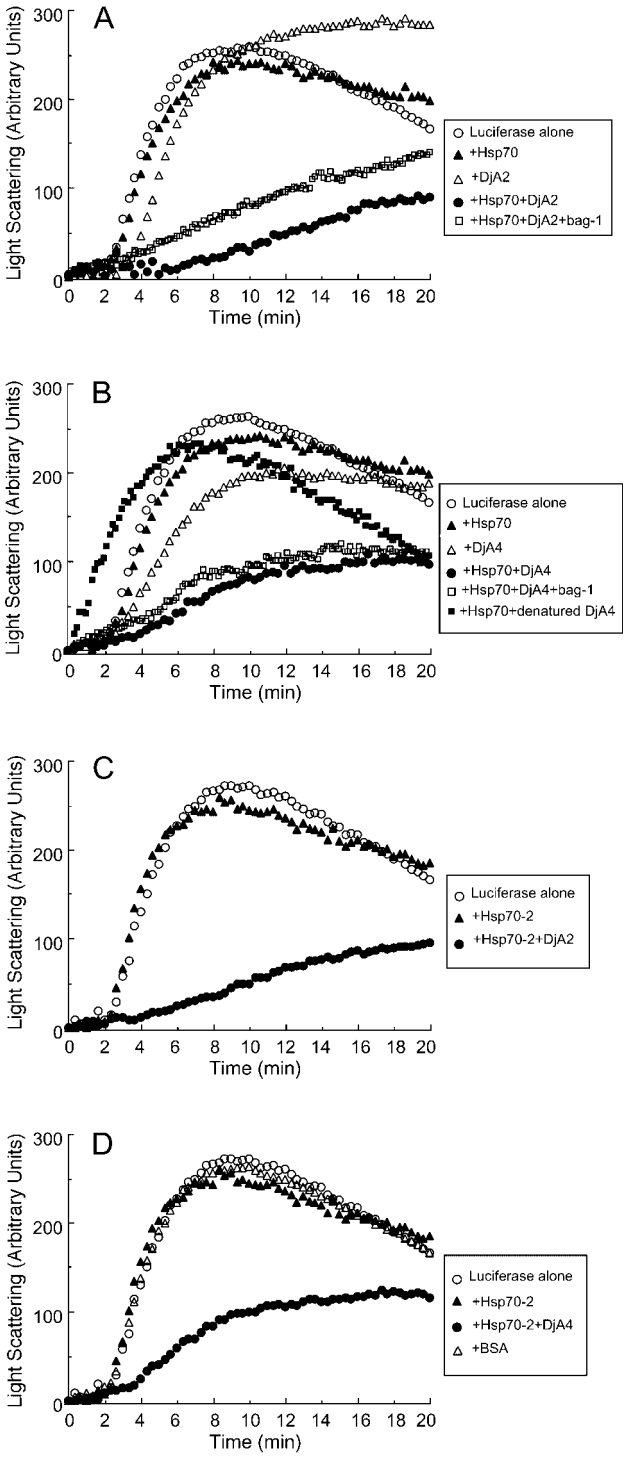

Fig. 5. **Effects of Hsp70 and DjA2 (A), Hsp70 and DjA4 (B), Hsp70-2 and DjA2 (C), and Hsp70-2 and DjA4 (D) on thermal aggregation of luciferase at 43°C.** The thermal aggregation of luciferase (12 μg/ml, 0.2 μM) was monitored in the absence (luciferase alone) or presence of the indicated chaperones by measuring the light scattering of the reaction mixtures at 320 nm. When heat denatured DjA4 plus Hsp70 were added, background scattering at zero time was twice as high as native DjA4 plus Hsp70. Final concentrations of chaperones were: Hsp70 and Hsp70-2, 0.5 μM; DjA2, DjA4, and bag-1, 0.2 μM.

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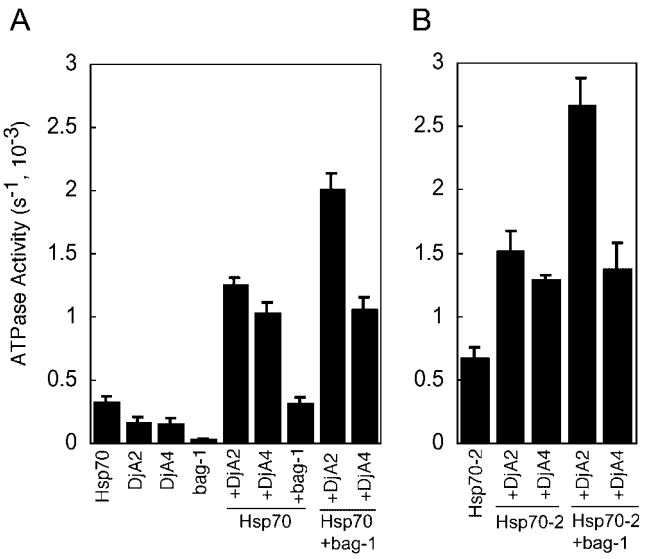

Fig. 6. **Effects of cochaperones on the ATPase activities of Hsp70 (A) and Hsp70-2 (B).** ATPase activity was measured for 60 min at 25°C in the presence of the indicated chaperones, and the results are presented as the mean ± SD (n = 3). Concentrations of chaperones were the same as in Fig. 3.

aggregation started a little later and proceeded similarly to the control after that (Fig. 5A). No decreased in light scattering was observed after 10 min, suggesting that DjA2 prevents the formation of larger aggregates. In sharp contrast, aggregation was markedly suppressed by Hsp70 plus DjA2. This suppression was partly canceled by bag-1. Similar results were obtained for the Hsp70 and DjA4 pair (Fig. 5B). Aggregation was moderately suppressed by DjA4 alone and more strongly by Hsp70 plus DjA4. In addition, heat denatured DjA4 together with Hsp70 did not prevent aggregation. Furthermore, the addition of denatured DjA4 together with Hsp70 instantly started luciferase aggregation. All these data suggest that functional DjA4 prevents luciferase aggregation, and bag-1 has little effect on the Hsp70–DjA4 pair.

The effects of Hsp70-2 and DjA2 or DjA4 on luciferase aggregation are shown in Fig. 5 (C and D). Hsp70-2 alone had little effect on aggregation. However, aggregation was strongly suppressed by the combination of Hsp70-2 plus DjA2 or Hsp70-2 plus DjA4. DjA2 in combination with Hsp70-2 was somewhat more effective than DjA4 in suppressing aggregation. The presence of an unrelated protein, bovine serum albumin (BSA), did not affect luciferase aggregation even at 100-fold molar excess over luciferase (Fig. 5D).

These results indicate that both DjA2 and DjA4 in combination of Hsp70 or Hsp70-2 are effective in preventing luciferase aggregation.

Effects of DjA2, DjA4, and bag-1 on the ATPase Activity of Hsp70 and Hsp70-2—Stimulation of the ATPase activity of Hsp70s is one of the notable properties of DnaJ family members. We analyzed the effect of DjA2 and DjA4 on the ATPase activities of Hsp70 and Hsp70-2 in the presence or absence of bag-1. ATP hydrolysis by Hsp70 or Hsp70-2 was almost linear up to 60 min (data not shown). The intrinsic ATPase activities of purified Hsp70 and Hsp70-2 were 0.322×10^{-3} s^{-1} and 0.674×10^{-3} s^{-1}, respectively. A similar value has been reported for Hsp70 (*29*).

As shown in Fig. 6A, the ATPase activity of Hsp70 was increased by 3.9-fold and 3.2-fold in the presence of DjA2 and DjA4, respectively. This indicates that both DjA2 and DjA4 are effective in stimulating the ATPase activity of Hsp70. bag-1, on the other hand, did not affect the ATPase activity. The ATPase activity of Hsp70–DjA2 was further stimulated by bag-1, whereas that of the Hsp70–DjA4 was not.

Similar results were obtained when Hsp70 was replaced by Hsp70-2 (Fig. 6B). The ATPase activity of Hsp70-2 was stimulated by both DjA2 and DjA4. The ATPase activity of the Hsp70-2–DjA2 pair was further enhanced by bag-1, whereas that of the Hsp70-2–DjA4 pair was not. In parallel experiments with Hsc70, the ATPase activity of Hsc70 was 0.933×10^{-3} s^{-1}, consistent with our earlier report (*25*). The ATPase activity of Hsc70 was stimulated by DjA4, but bag-1 did not accelerate that of the Hsc70–DjA4 pair (data not shown). The reason for this difference between DjA2 and DjA4 remains to be studied.

DISCUSSION

In this report, we focus on the cochaperone activities of type I DnaJ homologs, DjA2 and DjA4, on Hsp70s in luciferase refolding, ATP hydrolysis, and the prevention of protein aggregation. These cochaperone activities of DjA2 against Hsc70 (*25*), Hsp70 and Hsp70-2 (this study) are similarly positive. In contrast, DjA4 is active in the stimulation of ATP hydrolysis and in the prevention of protein aggregation with all Hsp70s, whereas it is inactive in luciferase refolding. These results indicate that these two type I DnaJs are not functionally equivalent.

Amino acid alignment of DjA1, DjA2, Ydj1, and DjA4 revealed two unique charged residues (E195 and K332) in DjA4. These positions in the other three eukaryotic type I DnaJs are conserved. The position of E195 corresponds to lysine, that of K332 to asparagine. The former position resides in the zinc-finger domain, and the latter in the C-terminal domain. Both domains are important for substrate binding (*1*, *7*). These charged residues may be responsible for the unique cochaperone activity of DjA4.

In mouse testis, the DjA4 protein is present in germ cells, and its expression is markedly induced as spermatogenesis proceeds (Fig. 1). The highest expression of DjA4 was found in elongated spermatids. The expression of Hsp70 is reported to be different from that of DjA4; Hsp70 is abundant in Leydig cells, but is negligible in germ cells (*30*). On the other hand, Hsp70-2 is expressed preferentially in spermatocytes and round spermatids (*27*), and is a candidate partner of DjA4. Because DjA1 and DjA2 are also expressed in spermatocytes (unpublished observation), DjA4 may act differentially from these two potent cochaperones.

Although DjA4, as well as DjA2, enhances ATP hydrolysis by Hsp70s, the further addition of bag-1 has no effect on ATP hydrolysis. We also found that bag-1 accelerates the refolding of denatured luciferase by Hsp70s–DjA2 pairs, but not by Hsp70s–DjA4 pairs. These observations

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suggest that the conformation of ADP-bound Hsp70s induced by DjA4 differs from that induced by DjA2. bag-1 has been reported to be a negative (*31–34*) or positive (*25*, *34*) regulator of Hsp70s in the refolding of denatured protein substrates. The different effects of bag-1 on the refolding activity of Hsp70s depend on the different DnaJ homologs. Despite difference in DnaJs, bag-1 further accelerates the ATPase activity of Hsp70s in all cases. Thus, DjA4 has distinct effects on Hsp70s and does not respond to bag-1. One possibility is that DjA4 alters the conformation of Hsp70s so that the subsequent binding of bag-1 or its function is impaired. The other is that DjA4 remains bound to Hsp70s and occupies bag-1 binding site(s). Further analysis of Hsp70s–DjA4 will provide novel mechanistic insights into the Hsp70-based chaperone system.

Results of the aggregation suppression assay reveal that both DjA2 and DjA4 are effective with Hsp70s in preventing luciferase aggregation (Fig. 5). bag-1 partly cancels the suppression activity of Hsp70s–DjA2, which may be caused by the subsequent release of denatured substrate protein after nucleotide exchange. Substrates released from Hsp70s–DjA2 have a higher tendency to form aggregates. Coincident with the results of the ATPase assay mentioned above, bag-1 did not affect the suppression activity of Hsp70s–DjA4 pairs.

DjA2 and DjA4 alone somewhat suppressed the aggregation of equimolar luciferase, while Hsp70 and Hsp70-2 alone had little effect, even at a 2.5-fold molar excess. These results suggest that DnaJs bind first to the denatured substrate. This is in accord with the findings that bacterial DnaJ binds to denatured substrates first, and then DnaK forms a ternary complex with the former two components (*35*, *36*). All type I DnaJs, including bacterial DnaJ (*37*), yeast Ydj1 (*15*), and mammalian DjA1, DjA2, and DjA4 (*38*, *39*, this study), suppress the aggregation of unfolded substrates. Interestingly, DjB1 had no effect on luciferase aggregation (data not shown). DjB1 has also been reported not to suppress the aggregation of rhodanese (*38*) and a CFTR-domain mutant (*39*).

The zinc-finger domain of type I DnaJs mediates their binding to unfolded polypeptides (*40*, *41*). Very recently, the zinc-finger domain of DnaJ was shown to be essential for binding to unfolded substrates and for the suppression of aggregation (*42*). Lack of the zinc-finger domain in other subfamilies of DnaJs (type II and type III) may reflect a difference in the cochaperone activities of DnaJs in protein refolding and suppression of aggregation.

This work was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (14037257 to M.M.) and grants from the Sumitomo Foundation (to K.T.) and the Naito Foundation (to K.T.).

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