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Isolation and characterization of a DnaJ-like protein in rats: The C-terminal 10-kDa domain of hsc70 is not essential for stimulating the ATP-hydrolytic activity of hsc70 by a DnaJ-like protein

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Abstract

A DnaJ-like protein, RDJ1, was isolated from a rat brain cDNA library. The protein is predicted to have 397 amino acid residues and shares 99% identity to that of HDJ2, a human DnaJ-like protein. *RDJ1* was also shown to rescue the temperature-sensitive lethality of a strain containing a mutated cytosolic DnaJ in yeast, *ydj1-151*. Fragments containing the J-domain of RDJ1 either with or without the G/F motif were expressed in *Escherichia coli*. The purified proteins stimulated the ATPase activity of hsc70 and of the 60-kDa N-terminal fragment of hsc70. These results imply that RDJ1 can interact with the N-terminal 60-kDa fragment of hsc70 to activate ATP hydrolysis by hsc70.

Keywords: ATPase of hsc70; DnaJ homolog; molecular chaperone; protein–protein interaction

The 70-kDa heat shock cognate protein (hsc70) is an ATP-dependent molecular chaperone and is thought to be involved in a variety of cellular processes such as translocation of polypeptides into organelles and folding of proteins (Gething & Sambrook, 1992; Georgopoulos & Welch, 1993; Hartl, 1996). To exert these functions, hsc70 has to work in concert with other cellular proteins and to interact with its substrates, which can be either nascent or unfolded polypeptides. Indeed, hsc70 has been shown to form complexes with both nascent polypeptides (Beckmann et al., 1990; Frydman et al., 1994) and unfolded proteins (Palleros et al., 1991; Sadis & Hightower, 1992). Several proteins have been identified to work together with hsc70 to exert the chaperone function. For instance, in *Escherichia coli*, DnaK (an hsc70 homolog) cooperates with DnaJ and GrpE to fold nascent polypeptides (Hendrick et al., 1993), repair proteins damaged by thermal stress (Schroder et al., 1993), and prevent denatured proteins from aggregating (Langer et al., 1992). Moreover, the ATPase activity of DnaK can be stimulated by DnaJ (Georgopoulos, 1992). GrpE alone plays little role in stimulating the ATPase activity of DnaK, but it accelerates the exchange of ATP for ADP on DnaK (Liberek et al., 1991). Recently, several DnaJ-like proteins have been identified in eukaryotes (Caplan et al., 1993; Cyr et al., 1994). These DnaJ-like proteins also bring about a tenfold stimulation of the ATPase activity of hsc70, implying that they indeed interact with hsc70 (Cyr et al., 1992; Cheetham et al., 1994; Minami et al., 1996).

Both hsc70 and the DnaJ-like proteins are composed of several functional domains. For hsc70, the N-terminal 44-kDa domain is an ATPase (Chappell et al., 1987; Flaherty et al., 1990; Huang et al., 1993), and the C-terminal 30-kDa domain is responsible for substrate binding. Recently, it was shown that an 18-kDa subdomain at the N-terminal region of the 30-kDa domain contains a binding site for short peptides (Wang et al., 1993; Zhang & Walker, 1996; Zhu et al., 1996). However, the entire C-terminal 30-kDa domain appears to be required for forming a tightly associated complex with S-carboxymethyl-α-lactalbumin (CMLA), an unfolded polypeptide (Hu & Wang, 1996).

All DnaJ-like proteins contain a highly conserved N-terminal region (ca. 70 amino acid residues) known as the J-domain. Following the J-domain, there is a glycine/phenylalanine (G/F)-rich region (ca. 40 residues), a cysteine-rich domain (ca. 80 residues), and a C-terminal motif containing approximately 170 amino acid residues (Cyr et al., 1994). A recombinant DnaJ fragment containing both a J-domain and a G/F-region (J-G/F) stimulates ATP hydrolysis by DnaK to a similar extent as intact DnaJ (Wall et al., 1994; Szabo et al., 1996). The cysteine-rich region forms a zinc finger structure (Banecki et al., 1996; Szabo et al., 1996) and is involved in binding denatured proteins (Szabo et al., 1996). This result is consistent with the observation that DnaJ itself appears to have chaperone activity (Hendrick et al., 1993). The functional significance of the C-terminal region has yet to be determined. The

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eukaryotic DnaJ-like proteins can also be resolved into these structural domains, except that in a few cases a cysteine-rich region is absent (Ohtsuka, 1993). It is generally accepted that the function of the equivalent domains between DnaJ and eukaryotic DnaJ-like proteins are similar, if not identical.

To define further how hsc70s and their cognate DnaJ-like proteins interact, we report here the identification and characterization of a new DnaJ-like protein, RDJ1, in rat. The protein can replace the function of Ydj1p (Caplan & Douglas, 1991; Atencio & Yaffe, 1992), a DnaJ homolog in *Saccharomyces cerevisiae*. Moreover, RDJ1 effects a fivefold increase in the ATPase activity of the N-terminal 60-kDa fragment of hsc70. This result implies that RDJ1 interacts with the N-terminal region of hsc70 to stimulate the ATP-hydrolytic activity of hsc70, and that the C-terminal 10-kDa fragment of hsc70 does not appear to be essential for this stimulation.

Results

On the basis of the nucleotide sequence of a human DnaJ-like protein, HDJ2 (Chellaiah et al., 1993; Oh et al., 1993), we designed a pair of primers to amplify by RT-PCR a rat cDNA fragment corresponding to both the J-domain and the G/F motif (J-G/F). Using this fragment, we screened a rat brain cDNA library and obtained several positive clones. The longest insert in these clones contained an open reading frame of 1,191 nucleotides encoding for a polypeptide of 397 amino acid residues. The deduced amino acid sequence of this DnaJ-like protein, *RDJ1* (Fig. 1), is virtually identical to that of HDJ2 and is 60% homologous and 48% identical to Ydj1p of *S. cerevisiae* (Caplan & Douglas, 1991; Atencio & Yaffe, 1992). We first investigated whether or not RDJ1 plays a functional role similar to that of Ydj1p in yeast. Plasmids with RDJ1 under the control of the *GAL1* promoter were transformed into a yeast strain containing a temperature-sensitive mutation in *YDJ1*. Then, we asked if the temperature-sensitive phenotype of the mutant could be reversed. As shown in Figure 2A, in the presence of galactose, yeasts harboring the plasmid with RDJ1 in the correct orientation grow at both the semi-permissive temperature (35 °C) and the nonpermissive temperature (37 °C). Indeed, under these conditions, the RDJ1 protein was expressed (Fig. 2B). These results imply that RDJ1 might functionally complement Ydj1p.

However, it might also be possible that expression of RDJ1 stabilizes the Ydj1-151p and brings about an increase in the level of Ydj1-151p in these cells, thus leading to rescue of the temperature sensitive phenotype. Therefore, we also examined the amount of Ydj1p by immunoblotting in this experiment. The results shown in Figure 2B demonstrate that the level of Ydj1-151p was unchanged regardless of whether the RDJ1 was present in the expression vector in either the forward or reversed orientation in galactose grown cells. Interestingly, however, there was somewhat more Ydj1p present in cells grown in glucose than in galactose medium, although again this was independent of the orientation of the RDJ1 gene (Fig. 2B). The basis for this carbon source-dependent change in Ydj1p levels is unknown. In any event, RDJ1 does not stabilize Ydj1-151p.

Because the activities of hsc70s may be modulated by DnaJ-like chaperones, we next studied if RDJ1 can stimulate the ATP-hydrolytic activity of hsc70. RDJ1 was expressed in bacteria with six consecutive histidinyl residues either at the N-terminus or at the C-terminus and was purified (Fig. 3A,B). The purified RDJ1 proteins were incubated with bovine hsc70 in the presence of ATP and the amount of ATP hydrolyzed was measured. The result

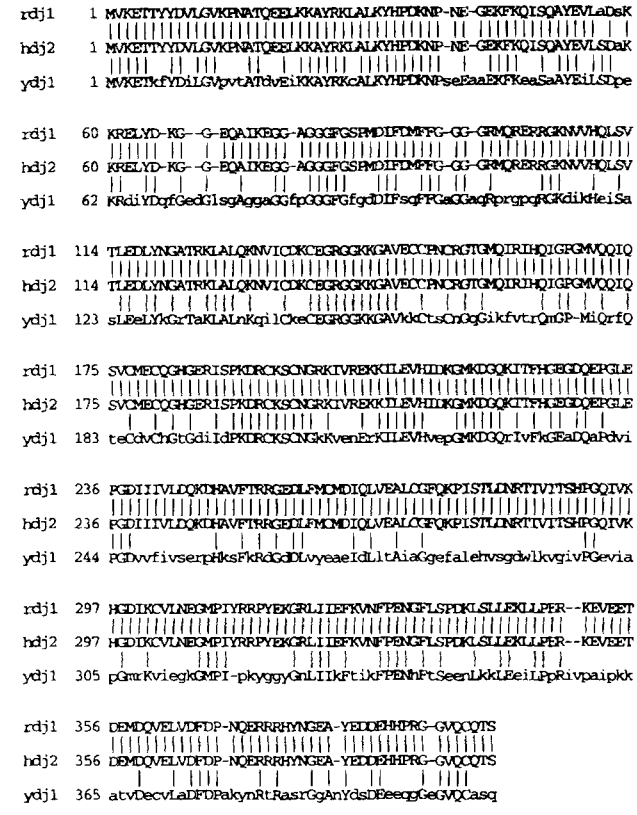

Fig. 1. Comparison of the predicted amino acid sequences of RDJ1 with HDJ2 and YDJ1. The amino acid sequence of a DnaJ-like protein of rats, RDJ1, was deduced from the cloned cDNA. The nucleotide sequence of this cDNA has been deposited in the EMBL Data Base under Accession Number U53922.

(Fig. 3C) demonstrates that RDJ1 causes a 15-fold increase in ATP hydrolysis by hsc70. On the other hand, incubating hsc70 with equivalent amounts of bovine serum albumin or lysozyme does not affect the ATP hydrolytic activity of hsc70 (data not shown). We then examined whether or not RDJ1 stimulates the ATPase activities of the 60-kDa and the 44-kDa N-terminal fragments of hsc70. The results (Fig. 3C) show that RDJ can simulate by fivefold the ATP-hydrolytic activity of the 60-kDa fragment. However, RDJ1 has little effect on ATP hydrolysis by the 44-kDa ATPase domain.

It has been shown that J-G/F domains are sufficient to stimulate the ATPase activity of DnaK or hsc70 (Wall et al., 1994; Szabo et al., 1996). In order to verify if this is also true for the equivalent fragment of RDJ1, we expressed and purified the J-G/F domains of RDJ1 (Fig. 4A). Then, we incubated purified J-G/F (Fig. 4B) with hsc70 and assayed for ATPase activity. The results shown in Figure 4C demonstrate that the J-G/F fragment of RDJ1 brings about a tenfold stimulation of ATP hydrolysis by hsc70. We also investigated if J-G/F activates the hydrolysis of ATP by the 60-kDa fragment. The results (Fig. 4C) indicated that J-G/F is also capable of increasing the ATPase activity of the 60-kDa fragment by sixfold. For comparison, stimulation of ATP-hydrolytic activities of hsc70 and of the 60-kDa fragment by CMLA is presented in Figure 4C. Evidently, J-G/F is much more effective than CMLA in activating the ATPase activities of both hsc70 and the 60-kDa fragment. The J-G/F concentration-dependent stimulation of ATP-hydrolytic activities of hsc70 and of the 60-kDa fragment was also

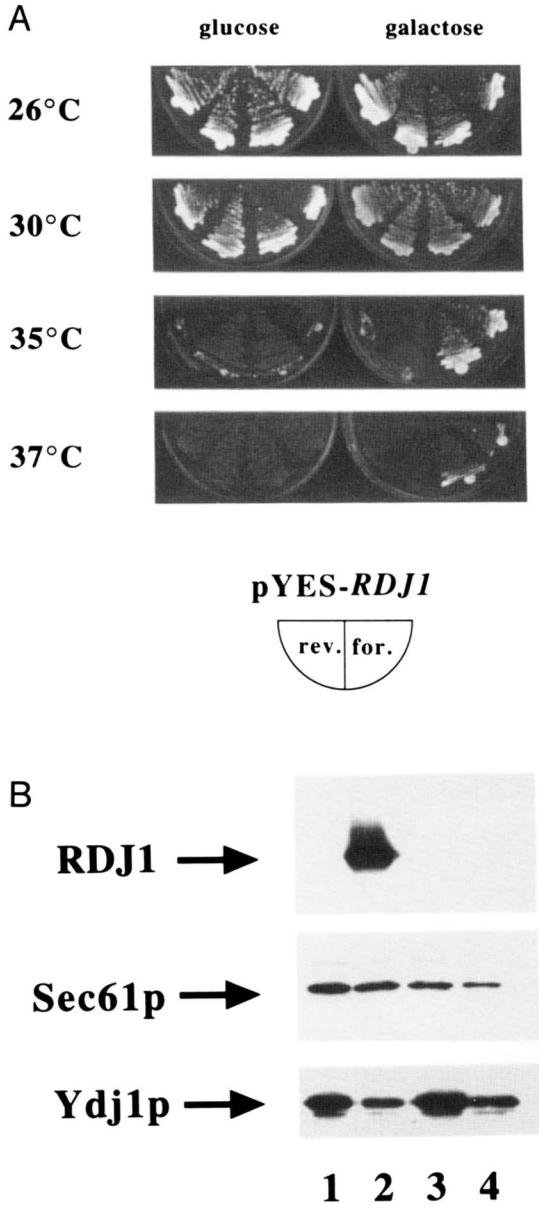

Fig. 2. Complementation of the *ydj1* temperature-sensitive mutant with RDJ1. **A:** Yeast strain ACY176 (*ydj1-151*) was transformed either with pYES2-RDJ1(for) or pYES2-RDJ1(rev) as described in Materials and methods. Transformants harboring the plasmids were plated onto uracil-free complete media containing either glucose or galactose and were incubated for two to three days at the indicated temperatures. **B:** Transformants generated in (A) were inoculated into the same liquid media either with glucose (lanes 1 and 3) or galactose (lanes 2 and 4). Extracts from yeast with both pYES2-RDJ1(for) (lanes 1 and 2) and pYES2-RDJ(rev) (lanes 3 and 4) were examined by immunoblotting with antibodies against J-G/F of RDJ1, Sec61p, or with anti-Ydj1p-antibodies.

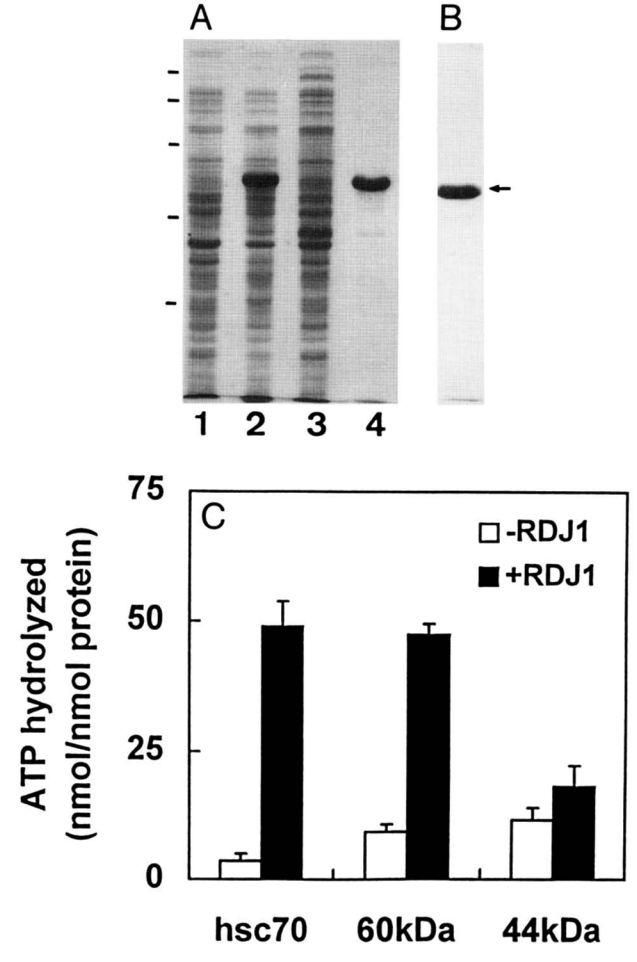

Fig. 3. Activation of Hsc70 ATPase by RDJ1. **A:** Bacteria were transformed with pRDJ-15b or pYES2-RDJ1(for) and the synthesis of RDJ1 was induced by IPTG. A Coomassie-stained gel is shown here. Lane 1 is a lysate from untransformed bacteria. Lanes 2 and 3 are the total extracts of bacteria with pRDJ-15b and pYES2-RDJ1(for), respectively, after induction. Lane 4 is RDJ1 purified by immobilized metal affinity chromatography. Molecular weight markers are β-galactosidase (116,000), phosphorylase b (97,000), bovine serum albumin (66,000), ovalbumin (45,000), and carbonic anhydrase (29,000). The RDJ1 with a $(His)_6$-tag at the N-terminus has an apparent mass of 55 kDa; whereas recombinant RDJ1 has a mass of 42 kDa. **B:** Purified RDJ1 with a $(His)_6$-tag at the C-terminus. The mobility of RDJ1 with the tag at the N-terminus on the same gel is indicated by the arrow. **C:** RDJ1 (4.5 μg) was incubated with 1 μg each of hsc70, the 60-kDa fragment and the 44-kDa ATPase domain with 0.2 mM [γ-^{32}P]-ATP at 37 °C for 60 min. The amount of ATP hydrolyzed was then measured. The result shown here was obtained with RDJ1 containing a C-terminal $(His)_6$-tag.

determined. As shown in Figure 5, with 1.1 μM of hsc70 and 1.2 μM of the 60-kDa fragment, the concentrations of J-G/F required to achieve half maximal stimulation of ATPase activities of hsc70 and the 60-kDa fragment are 2.2 and 2.3 μM, respectively. Interestingly, with similar concentrations of hsc70 or the 60-kDa fragment (one to two micromolar), a few hundred micromolar of peptide, with a length about 10 to 20 amino acid residues, are needed for half maximal stimulation of ATPase activity (Flynn et al., 1989; Fourie et al., 1994; Tsai & Wang, 1994).

Recently, the structure of J-G/F (residues 2–108) of DnaJ of *E. coli* was solved by NMR spectroscopy (Szyperski et al., 1994; Pellecchia et al., 1996). The results show that the J-domain is composed predominantly of four α-helices, but the G/F motif is relatively disordered. Therefore, J-G/F may resemble an unfolded polypeptide substrate to stimulate ATP hydrolysis by hsc70. To assess this possibility, we investigated whether or not J-G/F acts as a competitive inhibitor during CMLA/hsc70 complex formation. Since peptide substrates inhibit the formation of complexes be-

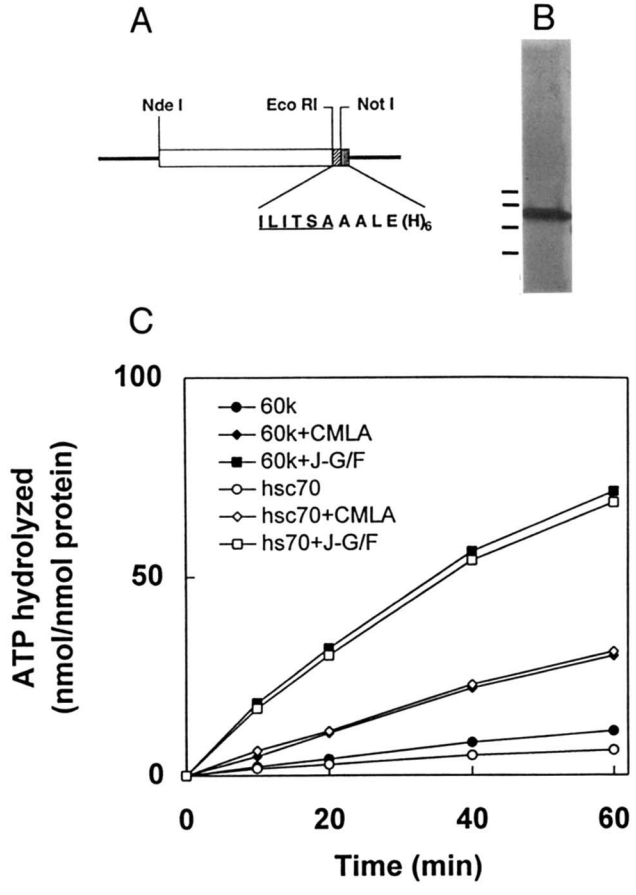

Fig. 4. Expression and characterization of J-G/F of RDJ1. **A:** The N-terminal fragment of RDJ1 composed of both the J-domain and G/F-rich region (J-G/F) was cloned into the pET-22b vector as described in Materials and methods. The J-G/F (open box) is followed by six amino acid residues (underlined) from the pGEM-T vector and then 10 residues including 6 consecutive histidines from pET-22b. **B:** The purified J-G/F was subjected to SDS gel electrophoresis with Tris/Tricine buffer. The J-G/F has an apparent mass of 12 kDa. The molecular weight markers are myoglobin (16,950), myoglobin I + II (14,440), myoglobin I + III (10,600), and myoglobin I (8,160). **C:** 1.6 μg hsc70 or 1.5 μg the 60-kDa fragment were incubated with 6 μg J-G/F or 20 μg of CMLA. Then, [γ-^{32}P]-ATP was added to the mixtures to initiate the reaction. At the time points indicated, the hydrolysis of ATP was measured as described in Materials and methods. In the presence of J-G/F and hsc70, about 25% of the ATP was hydrolyzed at the 60 min time point.

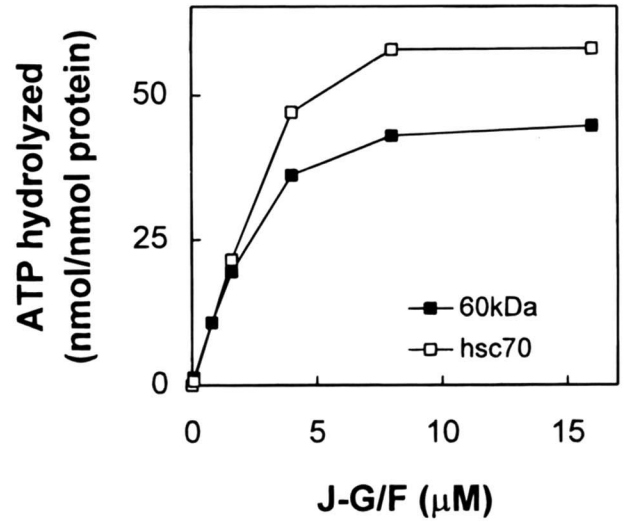

Fig. 5. J-G/F concentration-dependent ATPase activities of Hsc70 and the 60-kDa fragment. The ATP-hydrolytic activities of hsc70 and of the 60-kDa fragment were measured at various concentrations of J-G/F. The concentrations of hsc70 and the 60-kDa fragment were 1.1 and 1.2 μM, respectively, in a volume of 20 μL. The basal levels of ATP hydrolysis were subtracted.

tween hsc70 and CMLA (Fourie et al., 1994; Hu & Wang, 1996), one predicts that the level of hsc70/CMLA complexes should be reduced in the presence of J-G/F if J-G/F behaves as an unfolded polypeptide substrate. We, therefore, incubated hsc70 with CMLA in the presence of various concentrations of J-G/F. From the result shown in Figure 6 (lane 5), two additional bands appear after incubating hsc70 with CMLA. To identify the protein components in the two bands, we subjected each one to amino acid analysis. Two residues were obtained after each round of amino acid analysis for both the upper and lower bands. For the upper band, we obtained sequences that matches the N-termini of both hsc70 (SKG PAVGID) and CMLA (EQLTKcm-CEVF). For the lower band, we obtained the same sequence for hsc70 but the CMLA sequence was lacking the terminal glutamic acid. Interestingly, this lower band was not present when we used a different lot of commercially available CMLA (data not shown). Regardless, these results demonstrate that the indicated bands in Figure 6 represent *bona fide* complexes between hsc70 and CMLA. Moreover, the results shown in Figure 6 demonstrate clearly that J-G/F, over a concentration range of 0 to 50 μM, does not affect the formation of the complexes between hsc70 and unfolded polypeptides. Therefore, it is

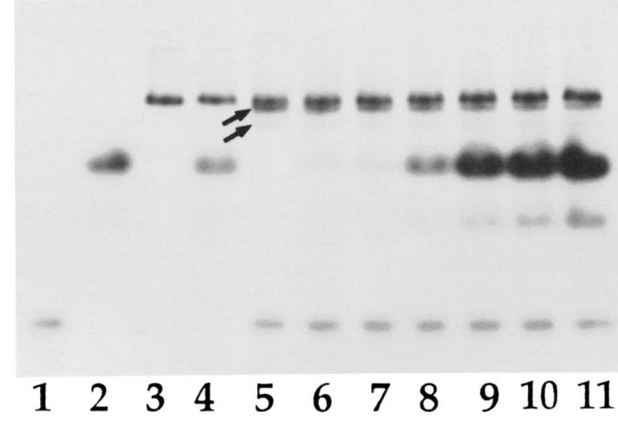

Fig. 6. J-G/F does not affect the formation of Hsc70/CMLA complexes. To determine if J-G/F would affect the formation of hsc70/CMLA complexes, various concentrations of J-G/F were incubated with bovine brain hsc70 (4.2 μM) and CMLA (15 μM) in a volume of 20 μL. Then, the mixtures were subjected to native gel electrophoresis. The hsc70/CMLA complexes (indicated with upper arrows) were visualized by staining the gels with Coomassie Brilliant Blue. This lot of CMLA (purchased from SIGMA) contains some degraded protein, which can also complex with hsc70 (lower arrow; see text for details). Lanes 1 and 2 are CMLA and J-G/F (20 μM) alone, respectively. Lane 3 is hsc70. Lanes 4 and 5 are hsc70 with J-G/F or with CMLA, respectively. Lanes 6 to 11 contain hsc70, CMLA, and 5, 10, 20, 30, 40, and 50 μM J-G/F, respectively.

unlikely that J-G/F simply mimics peptide substrates to stimulate the ATP-hydrolytic activity of hsc70. In addition, J-G/F does not appear to form tightly associated complexes with hsc70 (Fig. 6, lane 4).

If J-G/F does not act as an unfolded peptide to stimulate hsc70's ATPase activity, the question arises whether the J-domain alone affects the ATP-hydrolytic activity of hsc70. Results in the literature do not provide a clear answer to this question. On the one hand, the J-domain of *E. coli* DnaJ cannot stimulate the ATPase activity of DnaK (Wall et al., 1994; Szabo et al., 1996). At similar molar ratios, the J-domain of Hsp40 (HDJ1) is also inefficient in stimulating hsc70's ATPase activity (Minami et al., 1996). On the other hand, the J-domain of the cysteine string protein does activate the hydrolysis of ATP by hsc70 (Braun et al., 1996), and the J-domain of SV40 T-antigen stimulates the ATPase activity of both mammalian hsc70 and yeast Ssa1p (Srinivasan et al., 1997). Consequently, we decided to investigate whether or not the J-domain of RDJ1 can activate the ATP-hydrolytic activity of hsc70. The J-domain of RDJ1 was expressed as a fusion protein with a $(His)_6$-tag at the C-terminus and was purified (Fig. 7A). The purified polypeptide has an apparent mass of 11 kDa. However, the result from mass spectrometry analysis (Fig. 7B) demonstrates that these purified polypeptides contain two major species with masses of 9,658 and 9,527 Da. These two values are equal to those predicted for J-domain fusion proteins of RDJ1 with or without the initiating methionine. We then incubated various concentrations of purified J-domain fusion protein with hsc70 or with the 60-kDa fragment and subsequently measured the hydrolysis of ATP. The results (Fig. 7C) show clearly that the J-domain fusion protein alone is capable of increasing the amount of ATP hydrolyzed both by hsc70 and the 60-kDa fragment. Nevertheless, the concentrations of the J-domain protein required to stimulate ATP hydrolysis are significantly higher than those of J-G/F.

Conceivably, the $(His)_6$-tag located at the C-terminal region of the J-domain fusion protein may be unstructured, and it might mimic J-G/F to increase the ATP-hydrolytic activities of hsc70. To determine if this was the case, we digested the purified J-domain fusion protein with V8 protease to remove the $(His)_6$-tag, and then examined the effect of the J-domain lacking the tag on hsc70 ATPase activity. From the results shown in Figure 8A, after protease digestion, there is a reduction in the relative amount of J-domain fusion protein with a concomitant increase of a polypeptide with less mass. The products were then analyzed by mass spectrometry and were found to be composed of several different species. The major products with masses of 8,836 and 8,705 Da were cleaved at Glu-76, whereas the products with masses of 8,594 and 8,462 Da were cleaved at Glu-74. Moreover, a 8,106 Da species, corresponding to cleavages at both Glu-4 and Glu-76, was also detected. The proteolytic cleavage sites are summarized in Figure 8B. (One should notice from Fig. 7B that the J-domain fusion protein is composed of two distinct mass species with or without the initiating methionine.) Thereafter, we investigated whether or not these cleavage products, containing only the J-domain, remain capable of stimulating hsc70 ATPase. The results (Fig. 8C) show that they indeed increase the hydrolysis of ATP both by hsc70 and by the 60-kDa fragment.

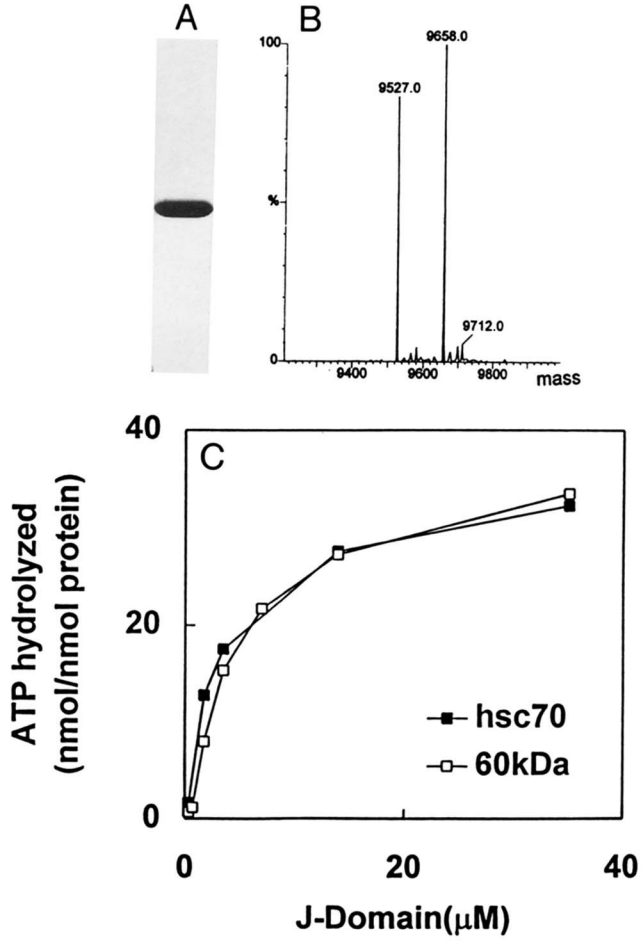

Fig. 7. Purification and characterization of the J-domain fusion protein. **A:** The J-domain was expressed with a $(His)_6$-tag at the C-terminus and purified by HisBind resin. The purified protein was displayed using Tricine gels. The J-domain fusion protein has an apparent M_r of 11,000. The molecular weight markers are myoglobin (16,950), myoglobin I + II (14,440), myoglobin I + III (10,600), and myoglobin I (8,160). **B:** The electrospray mass spectra of the J-domain fusion protein is shown. There are two major species corresponding to those with (M_r = 9,527) or without (M_r = 9,658) cleavage of the initiating methionine. **C:** One micromolar of hsc70 or the 60-kDa fragment was incubated with different concentrations of J-domain fusion protein with [γ^{32}P]-ATP for 60 min at 37 °C and ATP hydrolysis was measured.

Discussion

Herein, we have identified a rat DnaJ-like protein, RDJ1, that might be functionally equivalent to Ydj1p in *S. cerevisiae* since it is capable of rescuing the temperature-sensitive phenotype of the yeast *ydj1* mutant. However, attempts to determine whether or not RDJ1 could rescue the slow growth phenotype of a *ydj1* deleted strains, ACY1 (Caplan & Douglas, 1991) and MYY406 (Atencio & Yaffe, 1992), have been unsuccessful as we found that Δ*ydj1* cells containing the RDJ1 expression plasmid cannot recover from stationary phase when grown on galactose and minimal media, although the strain is Gal^+. It is interesting to note that strains deleted for *ydj1* are either inviable or grow very slowly (Caplan & Douglas, 1991; Atencio & Yaffe, 1992). It is possible that *YDJ1* is thus essential for cells to recover from growth arrest under poor nutrient conditions and that other genes may play a role in this complex process; in accordance with this view, molecular chaperones are known to be essential for cell growth recovery from adverse conditions (for example, see Parsell & Lindquist, 1994).

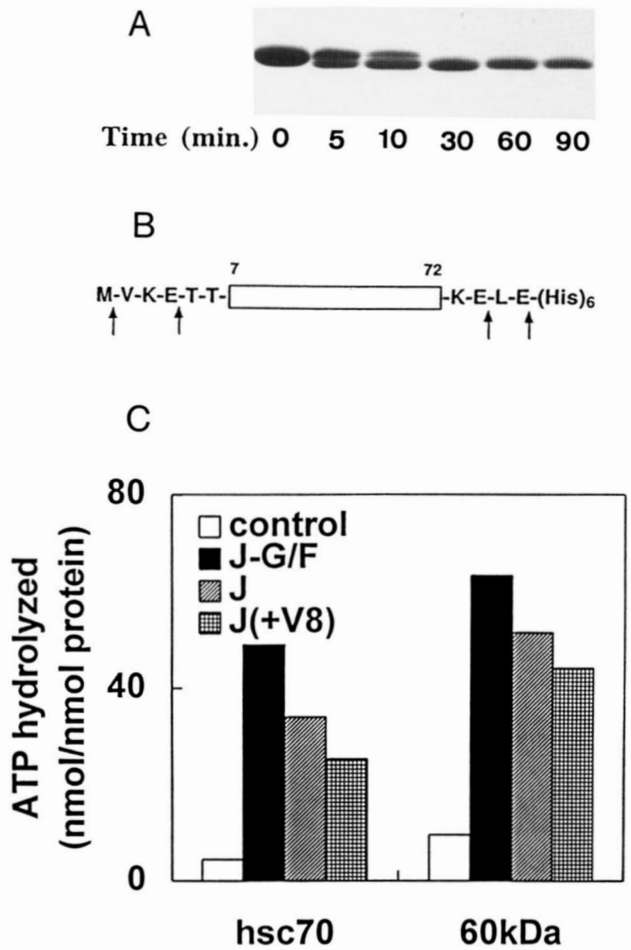

Fig. 8. Activation of Hsc70 ATPase by the J-domain of RDJ1. **A:** J-domain fusion protein was mixed with Sepharose coupled with *S. aureus* V8 protease. At the various time points indicated, an aliquot was withdrawn for SDS Tricine gel electrophoresis. **B:** After digesting for 90 min, the products were subjected to electrospray mass spectrometry analysis and the cleavage sites were deduced (indicated by the arrows). **C:** The hydrolysis of ATP was quantified after incubating hsc70 or the 60-kDa fragment with the protease-treated J-domain, J(+V8), for 60 min at 37 °C. The activation of ATP-hydrolytic activity by J-G/F or J-domain fusion protein is also given for comparison. The left and the right four columns are the results for hsc70 and the 60-kDa fragments, respectively. The control represents ATP hydrolysis without adding J-G/F, the J-domain fusion protein (J), or the V8-treated J-domain. The amounts of hsc70 and the 60-kDa fragments used in these experiments are 18 and 23 pmol, respectively. The molar ratios of the polypeptides are hsc70 : J-G/F = 1 : 10; hsc70 : J = 1 : 15; hsc70 : J(+V8) = 1 : 15; 60-kDa : J-G/F = 1 : 9; 60-kDa : J = 1 : 11; 60-kDa : J(+V8) = 1 : 11.

Clearly, the *ydj1-151* strain used in these experiments either does not lack these other genes or contains enough Ydj1p residual function so that this arrest phenotype is not evident (Fig. 2A).

We also show that the recombinant RDJ1, as well as its fragment composed of both J-domain and G/F motif (J-G/F), are capable of increasing the ATP-hydrolytic activity of hsc70 by at least tenfold. A similar level of stimulation was achieved previously by DnaJ or DnaJ-like proteins acting on DnaK, or hsc70 homologs (Georgopoulos, 1992; Cyr et al., 1992; Cheetham et al., 1994; Minami et al., 1996). On the other hand, peptide or unfolded protein substrates usually effect only a twofold to threefold stimulation of the ATPase activity of hsc70 (Flynn et al., 1989; Fourie et al., 1994; Tsai & Wang, 1994). Therefore, J-G/F appears to resemble intact RDJ1 in stimulating the ATPase activity of hsc70. It is formally possible that the less ordered G/F motif in J-G/F (Szyperski et al., 1994; Pellecchia et al., 1996) might act as unfolded polypeptide substrate to activate hsc70 ATP-hydrolytic activity. This scenario, nevertheless, is unlikely since if it were acting as a substrate, J-G/F would block the formation of complexes between hsc70 and CMLA (Fig. 6; Fourie et al., 1994; Hu & Wang, 1996). Moreover, with a similar amount of hsc70, the EC_{50} value (the concentration causing half maximal stimulation) of J-G/F required to activate hsc70 ATPase is significantly lower than that of peptide substrates or unfolded proteins (Fig. 5). Thus, we conclude that the interaction of hsc70 with J-G/F mimics that of hsc70 with native RDJ1.

We further show that, at concentrations higher than those used with RDJ1 or J-G/F, the J-domain of RDJ1 alone stimulates the ATP-hydrolytic activity of hsc70. A similar conclusion was made for the J-domain of the cysteine string protein (Braun et al., 1996). Our result is not necessarily inconsistent with the observation of Minami et al. (1996), who showed that J-domain alone is not effective in activating hsc70 ATPase activity, since they used a lower molar ratio of J-domain to hsc70 in their experiments. Moreover, the interaction between J-domains of various DnaJs with their hsc70/hsp70 partners may also differ (Hartl, 1996).

Interestingly, RDJ1, J-G/F, or the J-domain alone of RDJ1 can stimulate the hydrolysis of ATP by the 60-kDa fragment of hsc70. Thus, one may conclude that the C-terminal 10-kDa fragment of hsc70 does not play an essential role in mediating the stimulation of hsc70 ATPase activity by RDJ1. Our result does not necessarily contradict the view that DnaJ interacts with the C-terminal region of hsc70 to exert chaperone function (Cyr et al., 1994; Wawrzynow & Zylicz, 1995; Zhu et al., 1996), since stimulation of ATP-hydrolytic activity of hsc70 may represent only one aspect of hsc70-RDJ1 interaction. However, our conclusion differs from the result obtained by Freeman et al. (1995), who reported that HDJ1 does not affect the ATPase activity of hsp70 once the last four residues of hsp70 are deleted. Suffice it to say, different systems were used in these two studies. HDJ1 stimulation of hsp70 ATPase is relatively small even at a molar ratio of 1:10 (see Fig. 11 in Freeman et al., 1995). In addition, the 60-kDa fragment might have a conformation more similar to that of native hsc70, while removal of the last four residues of hsp70 produces a significant change in protein conformation (Freeman et al., 1995).

In summary, we have isolated a rat DnaJ-like protein, RDJ1, which may be functionally equivalent to Ydj1p in *S. cerevisiae*. Moreover, RDJ1, J-G/F, or the J-domain alone activates the ATP hydrolysis by hsc70 and by the N-terminal 60-kDa fragment of hsc70. These results imply that RDJ1 interacts with the N-terminal region of hsc70 to stimulate hsc70 ATPase. Future studies will define further the structure-function relationship of RDJ-hsc70 interactions using both biochemical tools for the mammalian proteins and genetic techniques for *YDJ1* and its hsc70 partner.

Materials and methods

Isolation of RDJ1 cDNA

Two primers, A and B, were synthesized for reverse transcriptase polymerase chain reaction (RT-PCR) with total RNA from rat hearts as templates. The procedures of Chirgwin et al. (1979) was used to

isolate total RNA. The sequence of primer A (5′-ACATATGGTG AAAGAAACAACT) is identical to the cDNA sequence initiating HDJ2, a human dnaJ-like protein (Chellaiah et al., 1993; Oh et al., 1993), except that an Nde I site is introduced at the 5′-end. The sequence of primer B (5′-AGAATTCCTCCTCCTCCAA) is complementary to that of HDJ2 at the end of the G/F-region, except for the addition of an EcoR I site at the 5′-end. The RT-PCR products were cloned into the pGEM-T vector (Promega) and were sequenced to confirm that they were homologous to HDJ2. Then, the inserts were excised from the plasmid by double digestion with Nde I and EcoR I, [^{32}P]-labeled by random priming and used to screen a library of rat brain cDNAs cloned into pGAD10 vector (Clontech). A total of six positive clones was obtained. The nucleotide sequence encoding a rat DnaJ-like protein, RDJ1, was deduced by the dideoxynucleotide method using Sequenase version 2.0 (United State Biochemical Corporation).

Cloning, expression, and purification of the recombinant J-G/F and J-domain

After sequence comparisons, we found that the fragment composed of the J-domain and the G/F-region (J-G/F) obtained by RT-PCR contained no amino acid substitutions. Therefore, one of the clones with J-G/F inserted in the forward direction in the pGEM-T vector was digested with Nde I and Not I. (The Not I site is in the multiple cloning site of the pGEM-T vector.) The fragment obtained (ca. 315 bps) was directionally cloned into the pET-22b vector (Novagen) digested with the same two enzymes. The resulted plasmid, p(J-G/F)-22b, was transformed into *E. coli* BL21(DE3), and the synthesis of J-G/F fragment was induced by the addition of IPTG as described previously (Hu & Wang, 1996). Bacteria were harvested, lysed by osmotic shock, and centrifuged (Wang & Lee, 1993). The supernatant then was incubated with TALON (Clontech) and mixed at room temperature for 30 min with a ratio of 200 mL liquid culture per mL packed resin. At the end of the incubation, the mixtures were chilled on ice and poured into a column. All subsequent steps were carried out at 4 °C. The column was washed with 40 volumes of 120 mM NaCl, 25 mM Tris/HCl (pH 8.0), followed by 40 volumes of 120 mM NaCl, 25 mM Tris, 10 mM imidazole (pH 8.0). The polypeptides bound to the resin were then eluted with 200 mM imidazole, 120 mM NaCl, 25 mM Tris (pH 8.0). The eluates were precipitated with $(NH_4)_2SO_4$ at 80% saturation. The precipitates were dialyzed against 120 mM NaCl, 25 mM Tris/HCl, 1 mM EDTA (pH 7.0) and were stored at 4 °C until use.

In order to clone and express the J-domain (the first 75 amino acid residues) of RDJ1, we first amplified the corresponding cDNA fragment by PCR using primers A and C (5′-TCTCGAGCTCCTT AATCGCCTGCT). The 5′-portion of the primer C includes an Xho I site and the rest is complementary to the coding sequence of RDJ1 at the end of J-domain. The cDNA of RDJ1 was used as template. The PCR products (ca. 230 bps) were cloned into the pGEM-T vector and were sequenced. Then, using the NdeI and Xho I restriction sites, the cDNA of the J-domain was cloned into the pET-22b. The plasmid obtained, p(J)-22b, is suitable for expressing the J-domain fusion protein in *E. coli*. The procedures for induction of synthesis and purification of the recombinant polypeptides are the same as those for J-G/F fragment described above. To remove the $(His)_6$-tag, the J-domain fusion protein was incubated with immobilized *Staphylococcus aureus* V8 protease (Pierce) in 0.1 M $(NH_4)HCO_3$ at 37 °C. At various time points, a small aliquot was withdrawn and resolved by SDS-PAGE to determine the efficiency of the digestion.

Cloning, expression in E. coli, and purification of RDJ1

Three plasmids were constructed to express RDJ1 in bacteria. To engineer the first plasmid, we synthesized primer D (5′-TCTCGA GAGAGGTCTGACACTGAAC), which is complementary to the coding sequence of the last six residues at the C-terminus of RDJ1, and which contains an Xho I site at the 5′-end. Then, the cDNA of RDJ1 was amplified by PCR with primers A and D. The PCR products were cloned into the pGEM-T vector, sequenced, and then digested with Hind III and Xho I. (A Hind III site is located around Ala-51 of the J-domain.) The fragment obtained (ca. 1050 bps) was ligated into p(J)-22b digested with the two enzymes. The resulting plasmid, p(RDJ1)-22b, is suitable to express the RDJ1 fusion protein in bacteria. To construct the second plasmid, the cDNA of RDJ1 was amplified by primers A and E (5′-TGGATCC TTAAGAGGTCTGAC). Primer E is complementary to the coding sequence of the C-terminal region of RDJ1 protein with a BamH I site immediately after the stop codon. After PCR amplification, the products were cloned into the pGEM-T vector for sequence verification. Then, they were excised with Nde I and BamH I and cloned into the pET-15b vector (Novagen) using these two restriction sites. The resulting plasmid, p(RDJ1)-15b, was used to express the RDJ1 fusion protein with hexahistidine at the amino terminus. To generate the third plasmid, we first cloned the C-terminal region of RDJ1, Hind III–BamH I fragment of p(RDJ1)-15b, into pYES2 (Invitrogen) resulting in plasmid *RDJ1C*/pYES2. Then, the N-terminal region of RDJ1 was amplified by PCR with primers B and F. Primer F (5′-AAGCTTATGGTGAAAGAAAC TAC) is identical to the cDNA sequence initiating RDJ1, except that a Hind III site was introduced at the 5′-end. The PCR products were cloned into the pGEM-T vector. After verification of the nucleotide sequence, the Hind III fragment (ca. 140 bps) was isolated and ligated into RDJ1C/pYES2 previously digested with Hind III. The plasmid obtained with the insert in the correct orientation, pYES2-RDJ1(for), is suitable for galactose-inducible expression of RDJ1 in yeast or in bacteria with the T_7 promoter. pYES2-RDJ1(rev), with the insert in the opposite orientation, was used as a negative control.

Bacterial cytosol containing recombinant RDJ1 proteins with the $(His)_6$-tag was applied to HisBind resin (Novagen), and the proteins were recovered with IMAC-100 (0.5 M NaCl, 100 mM imidazole, 10% glycerol, 25 mM Tris, pH 7.9). RDJ1 with the $(His)_6$-tag at the N-terminus was used directly to assay for activation of hsc70 ATPase. The protein with the $(His)_6$-tag at the C-terminus was further purified through a DEAE column (Cyr et al., 1992) before use.

Complementation of RDJ1 for YDJ1 in S. cerevisiae

Yeast strain ACY176 (*ydj1-151*), which is temperature sensitive for growth (Caplan et al., 1992), was transformed with plasmids pYES2-RDJ1(for) or with pYES2-RDJ1(rev). The transformants were selected at 26 °C on complete medium containing glucose but lacking uracil and were replated onto the same media containing either glucose or galactose. The plates were incubated for two to three days at various temperatures. To verify that RDJ1 was expressed in these transformants, single colonies were picked and inoculated into liquid media lacking uracil and containing either

glucose or galactose. Cell extracts were prepared and examined by immunoblotting using antibodies against J-G/F of RDJ1 or Ydj1p (Caplan & Douglas, 1991). Anti-Sec61p antibodies (Stirling et al., 1992) were also used on a parallel immunoblot as a loading control.

ATPase assay

ATP-hydrolytic activity was measured by the procedure of Tsai and Wang (1994), except that the total volume used for the assays was 20 μL. Briefly, hsc70 or the 60-kDa fragment of hsc70 was incubated at 37 °C in 20 μL of 75 mM KCl, 40 mM HEPES, 4.5 mM $Mg(CH_3COO)_2$, pH 7.0, and 0.2 mM [γ-^{32}P]-ATP with or without RDJ1, the J-G/F fragment, the J-domain of RDJ1, or CMLA. At various time points, 0.3 μL of the reaction mixtures were withdrawn, spotted onto PEI-cellulose sheets and the chromatograms were developed in 1.0 M formic acid/0.5 M LiCl. The amount of ATP hydrolyzed then was quantified by PhosphoImager analysis (Molecular Dynamics).

Competition of Hsc70/CMLA complexes by J-G/F

The procedures described previously (Hu & Wang, 1996) were used to determine whether or not J-G/F inhibits the formation of hsc70/CMLA complexes. Briefly, hsc70 and CMLA were incubated with J-G/F at 37 °C for 1 h, and the mixtures were subjected to native gel electrophoresis. To visualize the complexes, the gels were stained with Coomassie Brilliant Blue.

Other methods

To prepare antiserum, a 0.8 mg portion of purified J-G/F in phosphate-buffered saline was emulsified with an equal volume of complete Freund's adjuvant (Difco) and injected subcutaneously into a rabbit. The rabbit was boosted with an equal amount of the polypeptide with incomplete adjuvant on day 42 and 84. Serum prepared from blood collected on day 98 was used in this study. Purification of bovine brain hsc70 and the recombinant N-terminal 60-kDa fragment of rat hsc70 was performed by the methods described previously (Hu & Wang, 1996). Tricine-SDS gel electrophoresis was carried out by following the procedures of Schagger and von Jagow (1987). To determine the N-terminal sequences of the electrophoresed proteins, the proteins were transferred to Immobilon P (Millipore). The blot was stained with Coomassie Brilliant Blue to visualize the protein bands, which were then excised and sequenced on an ABI peptide sequencer (Model 494). Liquid chromatography/mass spectrometry analysis of the J-domain fusion protein or its cleavage products was performed by the procedures of Yeh et al. (1996). Proteins quantification was by the method of Lowry.

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