A Novel Function of 14-3-3 Protein: 14-3-3 ζ Is a Heat-Shock-related Molecular Chaperone That Dissolves Thermal-aggregated Proteins

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The 14-3-3 proteins are highly conserved molecules that function as intracellular adaptors in a variety of biological processes, such as signal transduction, cell cycle control, and apoptosis. Here, we show that a 14-3-3 protein is a heat-shock protein (Hsp) that protects cells against physiological stress as its new cellular function. We have observed that, in *Drosophila* cells, the 14-3-3 ζ is up-regulated under heat stress conditions, a process mediated by a heat shock transcription factor. As the biological action linked to heat stress, 14-3-3 ζ interacted with apocytochrome *c*, a mitochondrial precursor protein of cytochrome *c*, in heat-treated cells, and the suppression of 14-3-3 ζ expression by RNA interference resulted in the formation of significant amounts of aggregated apocytochrome *c* in the cytosol. The aggregated apocytochrome *c* was converted to a soluble form by the addition of 14-3-3 ζ protein and ATP in vitro. 14-3-3 ζ also resolubilized heat-aggregated citrate synthase and facilitated its reactivation in cooperation with Hsp70/Hsp40 in vitro. Our observations provide the first direct evidence that a 14-3-3 protein functions as a stress-induced molecular chaperone that dissolves and renaturalizes thermal-aggregated proteins.

INTRODUCTION

The 14-3-3 proteins are ubiquitously expressed within eukaryotic cells, and seven isoforms, designated as β , γ , η , ζ , τ , ϵ , and σ , have been described previously (Aitken *et al.*, 1992). The diverse biological functions of 14-3-3 depend on their ability to interact with a variety of proteins. Their original function was described as activator of neurotransmitter synthesis (Ichimura *et al.*, 1988) and subsequently as regulators of signaling proteins, such as Raf-1 (Freed *et al.*, 1994; Fu *et al.*, 1994), Bcr-Abl (Reuther *et al.*, 1994), polyoma middle T antigen (Pallas *et al.*, 1994), Cdc25 (Conklin *et al.*, 1995), phosphoinositide 3-kinase (Bonney-berard *et al.*, 1995), FKBP12-rapamycin-associated protein (Mori *et al.*, 2000), and BAD (Zha *et al.*, 1996). One well-defined mechanism for the recognition of target proteins is the binding of 14-3-3 to phosphorylated serine residues within the conserved motifs of the target molecules (Mushinski *et al.*, 1996; Yaffe *et al.*, 1997).

In the mechanistic basis of 14-3-3 function, the formation of 14-3-3 dimerization has a profound role in the regulation of the target protein function as a scaffold protein. A dimer 14-3-3 binds a target molecule at two sites and confers a conformational change that results in modulating its enzymatic activity (Tzivion *et al.*, 1998; Obsil *et al.*, 2001), al-

though the 14-3-3 monomer possesses ligand binding activity. Scaffold proteins, such as Protein p6, exhibit molecular chaperone-like activities, because they trap misfolded ligands for refolding and trafficking (Zahn *et al.*, 1996; Abril *et al.*, 1999). In contrast, some studies have shown that 14-3-3 proteins function as regulators of mitochondria or chloroplast precursor proteins trafficking (Hachiya *et al.*, 1994; May and Soll, 2000). In this process, 14-3-3 proteins target newly synthesized mitochondrial precursors into the organelle in an ATP-dependent manner. We previously found that 14-3-3 proteins exert intrinsic activities of ATP hydrolysis (ATPase) and ATP synthesis in presence of physiological concentrations of 5 mM ATP and 0.5 mM ADP (Yano *et al.*, 1997), the catalytic properties of which are similar to those of heat-shock protein (Hsp)70 (Hiromura *et al.*, 1998; Wu *et al.*, 2004). It is noteworthy that, among mitochondrial precursor proteins, apocytochrome *c* stimulates the ATPase activity of 14-3-3 most prominently (Hachiya *et al.*, 1994). Furthermore, evidence has been presented that 14-3-3 proteins are induced in response to external and internal stresses, such as nerve injury, pathogens, hypoxia, and metabolic stress (Bae *et al.*, 2003; Namikawa *et al.*, 1998; Roberts *et al.*, 2002), whereas their mechanisms of action have not been clarified. These properties of 14-3-3 proteins seem to be associated with those of a molecular chaperone, but their potential induction by stress or role in stress tolerance, properties that are characteristic of well-known molecular chaperones, have not been clarified.

This study was performed to determine whether the adaptor molecule 14-3-3 is a stress protein that possesses typical chaperone properties preventing the aggregation of, and disaggregating stress-denatured proteins under, physiological conditions. To develop a deeper biological understanding of a 14-3-3 protein as a molecular chaperone, we studied its role under heat stress conditions in *Drosophila*. In the

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Abbreviations used: ChIP, chromatin immunoprecipitation; CS, citrate synthase; HSE, heat-shock element; HSF, heat-shock transcription factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RNAi, RNA interference.

present study, we first found that the expression of *Drosophila* 14-3-3 ζ , but not 14-3-3 ϵ , is significantly up-regulated by heat stress and that the induction is regulated by a heat shock transcription factor (HSF). During heat stress, *in vivo*, 14-3-3 ζ interacts with heat-generated insoluble apocytochrome *c* and resolubilizes it. Our observations suggest that *Drosophila* 14-3-3 ζ is a heat-inducible molecular chaperone. The chaperone activity of 14-3-3 ζ was also observed with another mitochondrial protein, citrate synthase (CS), *in vitro*. 14-3-3 ζ resolubilized heat-aggregated CS and further reactivated it in corporation with Hsp70/Hsp40 chaperones *in vitro*. These *in vivo* and *in vitro* observations establish the role of 14-3-3 protein as a molecular chaperone, particularly in the prevention and reversal of heat-induced protein aggregation.

MATERIALS AND METHODS

Materials

The mouse monoclonal antibody (mAb) against human 14-3-3 ζ , which cross-reacts with *Drosophila* 14-3-3 ζ , but not with ϵ , and against human 14-3-3 ϵ , which cross-reacts with *Drosophila* 14-3-3 ϵ , were obtained from IBL (Gunma, Japan). A polyclonal rabbit anti-14-3-3 ζ antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). mAbs against native (holo, 556432) and denatured (apo, 556433) cytochrome *c* were obtained from BD Biosciences Pharmingen (San Diego, CA). Human Hsp70, human Hsp40, DnaK, anti-DnaK mAb, anti-HSF antibody, and anti-Hsp90 rat mAb (SPA-835), which interacts with *Drosophila* Hsp83, were obtained from StressGen Biotechnologies (San Diego, CA). The anti-Hsp90 mouse mAb and bovine Hsp90 were obtained from sigma. Apocytochrome *c* was prepared from horse heart cytochrome *c* (type VI; Sigma-Aldrich, St. Louis, MO) by a method described previously (Fisher *et al.*, 1973). Porcine citrate synthase (CS) was purchased from Roche Diagnostics (Indianapolis, IN). Recombinant 14-3-3 ζ protein with a hexahistidine tag at the amino terminus was expressed and purified as described previously (Wakabayashi *et al.*, 2001).

Cell Culture

Drosophila Schneider 2 (S2) cells were grown in Schneider cell medium supplemented with 10% fetal calf serum and cultured at 27°C. The activity of mitochondrial dehydrogenase 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine cell death. The assay was carried out according to standard method. The reaction product was measured at A_{570} , and the relative viability of heat-treated cells over untreated cells was calculated.

Reverse Transcriptase-Polymerase Chain Reaction (PCR)

An RNeasy mini kit (QIAGEN, Valencia, CA) was used to isolate total RNA from S2 cells. RT-PCR was carried out using a One-Step RT-PCR kit (QIAGEN) according to the manufacturer's instructions. We used the following primer pairs to amplify the Hsp83, 14-3-3 ζ , 14-3-3 ϵ , and actin: Hsp83 GenPeptide, accession no. AE003477, forward primer (F) 5'-GGCTGATGATGAGAAGAAGGA-3' and reverse primer (R) 5'-CTCAATCAGCTCCATGGTCTT-3'; 14-3-3 ζ , accession no. AE003831, (F) 5'-GTCATCGTGCGGTGTCATCT-3' and (R) 5'-TCAACAGCTGCATGATGAGTG-3'; 14-3-3 ϵ , accession no. U84898, (F) 5'-TCGTGGCGCATCATCACCT-3' and (R) 5'-TGCATGATGAGTGTCTGAGTCT-3'; and actin, accession no. NM-078901, (F) 5'-TTGCTTACTGAGGCTCCTTTG-3' and (R) 5'-TGAGTTGTAGTGCTCTCGTG-3'. The products were examined by agarose gel electrophoresis after 21 cycles.

RNA Interference (RNAi)

DNA fragments for the target proteins were amplified by RT-PCR. The primer sequences used to generate specific double-stranded RNA (dsRNA) were obtained as follows: HSF, accession no. AE003800, (F) 5'-GCAACATGCTCGCGTGAA-3' and (R) 5'-TGTCGTCGTAGCTTGTGTGTC-3'. We used the primer pairs described above to generate specific dsRNAs for 14-3-3 ζ and Hsp83. T7 RNA polymerase binding site (TTAATACGACTCACTATAGG-GAGA) was added at the 5' end of each primer. The PCR products were used as templates, and dsRNA was synthesized using a MEGAscript kit (Ambion, Austin, TX) according to manufacturer's instructions. S2 cells adjusted to a final concentration of 1×10^6 cells/ml in *Drosophila* serum-free medium were plated into a six-well cell culture dish, to which 15 μ g of dsRNA was added, and the dish was incubated at room temperature (RT) for 1 h, followed by addition of Schneider medium. The cells were incubated for an additional 4 d to allow an effective decrease in the expression of the respective target molecules.

Preparation of Insoluble and Soluble Cell Fractions

Insoluble cell fractions were prepared according to a method described previously (Mogk *et al.*, 1999) with slight modifications. S2 cells treated or untreated with dsRNA were grown at 27°C and shifted to 37°C for 1 h, followed by incubation at 27°C for 0–3 h. The cells were collected by centrifugation at 4°C and washed in phosphate-buffered saline (PBS). The cell pellets were suspended in buffer A (100 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM EDTA, and 15% sucrose) and frozen at -80°C . After slow thawing of the samples at 0°C, the cells were sonicated on ice in a Branson Ultrasonics (50% duty; 4 cycles). After removing the nuclei and undisrupted cells by centrifugation at $2000 \times g$, the insoluble cell fraction was pelleted by centrifugation at $15,000 \times g$ for 15 min at 4°C and then washed twice. The supernatant was collected as a soluble fraction. The insoluble and soluble fractions, which contain both cytosol and mitochondrial proteins, were analyzed by immunoblotting.

Cell Fractionation

Subcellular fractionation was performed as described previously (Varkey *et al.*, 1999). S2 cells lysed in buffer B (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, and 2 mM EDTA) containing 0.25 M sucrose were homogenized in a Dounce homogenizer and centrifuged at $1000 \times g$ to separate nuclei and unbroken cells. The supernatants were centrifuged at $10,000 \times g$, for 15 min, and the pellets were collected as the heavy membrane/mitochondria fraction. The supernatants were further centrifuged at $100,000 \times g$, for 30 min, and the resulting supernatants were collected as the cytosol fraction.

Immunoblotting and Immunoprecipitation

S2 cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 1% NP-40, 0.5% deoxycholate, 0.4 mM EDTA, and 0.5 mM sodium orthovanadate) for 30 min at 4°C. Cell lysates, insoluble cell fractions, and immunoprecipitates were resolved in Laemmli sample buffer. Samples were subjected to SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, reacted with the respective antibodies, and detected with an enhanced chemiluminescence detection kit. Because antibody against holocytochrome *c* reacts with native cytochrome *c*, but not with denatured cytochrome *c*, immunoblotting for holocytochrome *c* was carried out using mAb against denatured cytochrome *c*.

For immunoprecipitation, soluble cell fractions, or the insoluble fractions dissolved with RIPA buffer, were incubated with the indicated antibodies for 1 h at 4°C. Protein G-Sepharose beads were then added to collect the immunocomplexes for an additional 1 h of incubation. The pellets were washed three times with RIPA buffer.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed as described in the EZ ChIP kit manual (Upstate Biotechnology, Charlottesville, VA). S2 cells grown to a density of 1×10^7 cells/ml, with or without heat treatment, were cross-linked to their DNA binding sites by adding formaldehyde to a final concentration of 1%, for 10 min. The cells were suspended in SDS lysis buffer, and the chromatin DNA was disrupted into fragment sizes of 200–1000 base pairs by sonication with 6×10 -s bursts on ice. Supernatants collected by centrifugation were diluted in ChIP dilution buffer. For the immunoprecipitation, the lysate was incubated with anti-HSF antibody overnight at 4°C, followed by immobilization on salmon sperm DNA/Protein G agarose (Upstate Biotechnology). IgG was used in immunoprecipitates as a control for nonspecific signals. The protein/DNA complexes extracted with elution buffer were heated to 65°C for 6 h to reverse cross-links and then digested with proteinase K (PK). DNA fragments were purified and amplified in PCR with the ChIP assay primers containing the heat-shock element (HSE) sites in each chaperone promoter. PCR primers for the ChIP were as follows: 14-3-3 ζ (–381/+37), (F) 5'-AAGCGCTC-GAAAGTTTCAACA-3' and (R) 5'-ACGAACACTACCAACGAACGA-3'; and hsp70Ab (–215/+54), (F) 5'-GGCAGAAAGAAAACCTCGAGAA-3' and (R) 5'-TTCAGCTGCGCTTGTGTTTGT-3'. 14-3-3 ϵ (–377/+20) was amplified with the following primers: (F) 5'-AGCTAGTGTGACCAAGGACG-3' and (R) 5'-TCCGTTTGTGTGAAGCTTTT-3' as a negative control for HSF target.

Acidic Native-PAGE

Acidic native-PAGE was performed by the modified method of Reisfeld *et al.* (1962). Horse heart cytochrome *c*, apocytochrome *c*, or S2 cell extracts without intact cells were loaded onto 4% acid polyacrylamide gel prepared in a 0.385 M acetic acid-KOH solution, pH 4.3. The electrophoresis was carried out with acidic running buffer (0.14 M acetic acid and 0.35 M β -alanine, pH 4.3) and a reverse polarity electrode, using methyl green as the tracking dye. The gel was blotted to a PVDF membrane with acidic transfer buffer and then developed with anti-apocytochrome *c*-specific antibody.

Immunofluorescence

The immunofluorescence of heat-treated or untreated S2 cells grown on glass chamber slides was analyzed. For the MitoTracker staining, cells were incubated with 400 nM MitoTracker Red (Invitrogen, Carlsbad, CA) for 15 min at

RT, fixed for 20 min in 4% paraformaldehyde in PBS, and permeabilized with 0.1% Triton X-100 for 10 min at RT. The cells were stained with anti-holo or -apocytochrome *c* mAb (10 μ g/ml) and anti-14-3-3 ζ antibody (5 μ g/ml) or anti-Hsp90 rat mAb (4 μ g/ml) diluted in blocking buffer for 2 h. The cells were washed in PBS and incubated for 1 h with either Alexa Fluor 488 (green)-conjugated goat secondary antibody against rabbit or rat IgG and Texas Red-conjugated goat secondary antibody against mouse IgG (Invitrogen). The stained cells were visualized on a laser scanning confocal microscope (TCS-NT; Leica, Wetzlar, Germany).

Proteinase K Susceptibility Assay

S2 cells treated with dsRNA-directed against 14-3-3 ζ were incubated at 37°C for 1 h. The insoluble cell fractions were prepared and then incubated in absence or presence of additional 14-3-3 ζ or bovine Hsp90 (10 μ g) in refolding buffer (50 mM HEPES-KOH, pH 7.5, 150 mM KCl, 5 mM MgCl₂, and 10 mM dithiothreitol [DTT]), containing 5 mM ATP at 27°C for 0–2 h. PK was then added to each reaction mixture to a final concentration of 10 μ g/ml and followed by incubation on ice for 10 min. The reactions were terminated with 2 mM phenylmethylsulfonyl fluoride and then subjected to SDS-PAGE followed by immunoblotting.

Disaggregation Assay by Centrifugation

Heat-insoluble S2 cell fractions were prepared and then incubated with or without 14-3-3 ζ or Hsp90 (10 μ g) in refolding buffer, in absence or presence of 5 mM ATP at 27°C, for 4 h. Aggregated proteins were pelleted by centrifugation for 20 min at 20,000 $\times$ g, washed twice and then dissolved with RIPA buffer and analyzed by immunoblotting using cytochrome *c*-specific antibody. CS (10 μ g) was incubated at 43°C for 5 min in a solution of 40 mM HEPES-KOH, at pH 7.5, for its aggregation. 14-3-3 ζ or Hsp90 (10 μ g) was added after heat treatment of CS and incubated in refolding buffer at RT for 4 h, in absence or presence of 5 mM ATP. Aggregated fractions (pellets) were separated by centrifugation and analyzed by silver staining.

Spectroscopic Measurements

Aggregation was monitored continuously as the increase in turbidity at 320 nm with an F-2000 fluorometer (Hitachi, Tokyo, Japan). The pH-dependent aggregation of purified apocytochrome *c* was assessed by adding apocytochrome *c* to the reaction buffer (40 mM HEPES-KOH) of pH 5.0 or 7.5 at RT. Thermal aggregation was carried out in a solution of 40 mM HEPES-KOH, at pH 7.5, heated to 48°C for apocytochrome *c*, and to 43°C for CS. To ascertain the prevention of protein aggregation by the chaperones, each substrate was diluted into a preheated reaction mixture containing various amounts of 14-3-3 ζ or Hsp90, and the light scattering was measured. For disaggregation assay, purified apocytochrome *c* was heated to 48°C for 10 min. Disaggregation reactions were performed at 25°C by adding aggregated apocytochrome *c* to a buffer containing 50 mM HEPES-KOH, pH 7.5, 5 mM MgCl₂, 5 mM ATP, and 10 mM DTT, in absence or presence of chaperones. Disaggregation of aggregated apocytochrome *c* was monitored for 30 min by light scattering.

Reactivation of Citrate Synthase

CS was incubated at 43°C for 15 min in a solution of 40 mM HEPES-KOH, at pH 7.5, to promote its aggregation. Protein refolding was started by adding aggregated CS to refolding buffer containing 2 mM ATP at 25°C, in absence or presence of the indicated chaperones. The activity of the reactivated CS was assayed spectrophotometrically at various time points with freshly prepared oxaloacetic acid and acetyl-CoA, according to a method described previously (Buchner *et al.*, 1998).

RESULTS

Heat Shock-related Expression of 14-3-3 ζ and Its Regulation at the Transcriptional Level

In contrast to vertebrates, which possess many 14-3-3 isoforms, *Drosophila* has only two isoforms (ζ and ϵ) and is therefore a simpler system in which to elucidate their physiological functions. *Drosophila* 14-3-3 ζ shares an 81% amino acid identity with its closest mammalian homologue, 14-3-3 ζ (Kockel *et al.*, 1997). We initially determined the expression pattern of 14-3-3 ζ and its stability in cells after heat treatment, because the heat-shock gene system found in *Drosophila* is a prominent model that induces several heat-shock proteins under heat stress conditions. S2 cells were heated up to 37°C for 1 h and then allowed to recover at 27°C for 1–3 h. The expressions of 14-3-3 ζ and Hsp83, a homologue of Hsp90 in *Drosophila* whose synthesis is known to increase severalfold under stress conditions, were examined by im-

munoblotting. The protein expression of 14-3-3 ζ as well as Hsp83 were significantly up-regulated and increased approximately twofold after heat treatment in comparison with those of the untreated cells, whereas there were no apparent increases in 14-3-3 ϵ and actin (Figure 1A, left and middle). We next examined whether up-regulation of 14-3-3 ζ by heat stress is observed in mRNA level. The results of semiquantitative RT-PCR analyses revealed that the levels of 14-3-3 ζ transcripts increased gradually after heat shock, reaching a peak at 1 h of recovery, whereas the expression of Hsp83 mRNA increased more rapidly after heat shock (Figure 1A, right). The transcript levels of 14-3-3 ϵ and actin were constant in disregard of heat treatment.

The induction of heat-shock proteins by various stresses is mediated by an HSF. In *Drosophila*, a single HSF encoded by a single-copy gene, which binds to an upstream DNA binding motif known as HSE, promotes the transcription of heat-shock genes (Fernandes *et al.*, 1994). The sequence of the promoter region of 14-3-3 ζ reveals that consensus motifs for HSF binding are located upstream of the transcriptional start site, although it is not conserved in the 14-3-3 ϵ gene. To determine whether the heat induction of the 14-3-3 ζ protein is regulated transcriptionally, we examined the effects of actinomycin D and the inactivation of HSF using RNAi on the induction of 14-3-3. Preincubation with actinomycin D prevented the increase in 14-3-3 ζ and Hsp83 protein expression caused by heat stress (Figure 1B). We then examined the effects of RNAi on *Drosophila* HSF. When the cells were treated with dsRNAs targeted to HSF, levels of HSF mRNA were significantly reduced, as determined by RT-PCR (Figure 1C). To confirm that the effect was not a nonspecific effect of RNAi, we also reduced the endogenous 14-3-3 ζ and Hsp83 levels from S2 cells by using RNAi and confirmed the specificity of the efficiency of RNAi. Marked and selective decreases in 14-3-3 ζ and Hsp83 proteins were observed after treatment with each respective dsRNA (Figure 1C). As a consequence of the HSF silencing, the expression of 14-3-3 ζ remained unchanged after heat treatment, compared with the expression of actin (Figure 1D). Reflecting the transcriptional regulation of Hsp83 by HSF (Fernandes *et al.*, 1994), HSF RNAi also abolished the induction of Hsp83. In contrast, cultures treated with 14-3-3 ζ dsRNA greatly induced the expression of Hsp83 after heat-stress (Figure 1D). Similar induction of 14-3-3 ζ was observed when cells were treated with Hsp83 dsRNA, suggesting the selective effect of HSF RNAi on the heat induction of 14-3-3 ζ or Hsp83. To examine the HSF-mediated transcription of 14-3-3 ζ under heat-stress conditions, the ability of HSF to bind to the 14-3-3 ζ promoter in vivo was determined by CHIP assay, using primers designed to span a proximal portion of the 14-3-3 ζ promoter containing the HSE site. Because clear association of HSF with the 14-3-3 ζ gene promoter was observed in heat-treated but not untreated S2 cells (Figure 1E). A control assay using primers specific to the *Drosophila* Hsp70(Ab) exhibited a promoter-specific pattern of heat-inducible DNA binding of HSF, whereas an interaction with HSF was not observed for the 14-3-3 ϵ promoter gene in heat-treated cells. These results indicate that 14-3-3 ζ is a heat-shock protein, the expression of which is regulated at the transcriptional level.

Apocytochrome *c*, a Mitochondrial Precursor Protein, Aggregates in the Cytosol of S2 Cells under Heat Stress Conditions

It has been proposed that the 14-3-3 proteins trap newly synthesized mitochondrial precursors and stimulate mitochondrial import (Neupert, 1997). Besides the function of 14-3-3 reported, we found a new function of 14-3-3 as a

Figure 1. Heat-induced expression of 14-3-3 ζ is regulated at the transcriptional level. (A) Heat induction of 14-3-3 ζ . S2 cells were incubated at 37°C for 1 h and allowed to recover for 1–3 h at 27°C. Cell extracts obtained at the indicated times (hours) were separated by SDS-PAGE and immunoblotted with an anti-Hsp90 (Hsp83), 14-3-3 ζ , or 14-3-3 ϵ antibody (left). Numbers at the bottom indicate hours of recovery after heat shock. The expression of actin was monitored as an internal control. Middle, quantification of each protein level in arbitrary unit (arb-u) estimated by densitometric analysis using Scion Image (Scion, Frederick, MD). The panels on the right indicate the heat-induced expression of 14-3-3 ζ mRNA. The alteration of each mRNA expression after heat shock was analyzed by semiquantitative RT-PCR. (B) Effects of actinomycin D on the synthesis of 14-3-3 ζ . S2 cells were preincubated for 30 min with 5 μ g/ml actinomycin D at 27°C before being exposed to heat shock and shifted to 37°C for 1 h, followed by incubation at 27°C for 1–3 h. Each extract was analyzed by immunoblotting. Nonheat-shocked samples with or without actinomycin D were prepared as controls for the heat-shock experiments. Expressions of 14-3-3 ζ , Hsp83 and actin in the cell extracts were monitored. (C) Effects of HSF dsRNA on the mRNA level of HSF was confirmed by semiquantitative RT-PCR analysis (top). The silencing efficiency of 14-3-3 ζ or Hsp83 RNAi was determined by immunoblotting with appropriate antibodies (bottom). (D) S2 cells treated with each dsRNA were exposed to 37°C for 1 h. The expression of 14-3-3 ζ , Hsp83 and actin in the cell extracts prepared after each specified recovery period (hours) were monitored by Western blot. Time is hours of recovery. (E) ChIP assay for the association of HSF with 14-3-3 ζ gene before and after heat shock. The DNA in the product immunoprecipitated by anti-HSF or nonimmune IgG was followed by PCR with primers specific to 14-3-3 ζ , Hsp70Ab, or 14-3-3 ϵ promoter. Actin signal is a control of DNA input (bottom left). The immunoprecipitates with antibody against HSF were confirmed by immunoblotting (bottom right). The results shown are representative of three separate experiments.

stress-related molecular chaperone under heat stress conditions. Because apocytochrome *c* is the most prominent precursor protein reported which enhances the ATPase activity of 14-3-3 proteins (Hachiya *et al.*, 1994), an investigation was

undertaken to determine the role of 14-3-3 ζ in apocytochrome *c* under heat-stress conditions. Apocytochrome *c* is synthesized in the cytoplasm as a precursor, then translocated into the mitochondrial intermembrane space, where it

Figure 2. Difference in the heat-induced change in intracellular localization among apo- and holo-cytochrome *c* in cells. S2 cells kept at 27°C or heat-treated at 37°C for 1 h were costained with Mito-Tracker Red and either anti-holo- (A) or apo-cytochrome *c* (B) antibody. After incubation with Alexa Fluor 488 (green)-coupled secondary antibodies, the cells were visualized by laser confocal immunofluorescence microscopy. Bottom, merging of both staining procedures. (C) Extracts from heat-treated or -untreated S2 cells were fractionated by centrifugation into mitochondria and cytosol. Individual fractions were visualized by immunoblotting using anti-cytochrome *c* antibody. The expression of grp75 (mitochondria) or extracellular signal-regulated kinase (ERK) (cytosol) was detected as a marker for proper cell fractionation. The cells treated with 20 μ g/ml cycloheximide for 2 h before heat stress were fractionated and analyzed as described earlier (right). (D) Absence of heat-induced apoptosis in S2 cells treated at 37°C for 1 h. Cell death was analyzed by MTT. The value was expressed as the percentage of cell viability without heat stress, which was set at 100%. Error bars represent the SD from triplicate trials. (E) Acidic native-PAGE followed by immunoblotting using an apocytochrome *c*-specific antibody. Lane 1, holo-cytochrome *c* (horse heart); lane 2, apocytochrome *c* (purified from holo-cytochrome *c*); and extracts prepared from untreated (lane 3) or heat-treated (lane 4) cells. Lane 2 shows where the monomeric apocytochrome *c* migrates. Note that apocytochrome *c* in heat-treated cells forms a high-molecular-weight product.

is converted to the holo-form by cytochrome *c* heme lyase (Nicholson *et al.*, 1988). We initially examined the possibility of apocytochrome *c* aggregation in the cytosol of S2 cells under heat stress conditions.

The subcellular localization of cytochrome *c* in the cells treated with or without heat stress was determined by immunofluorescence microscopy (Figure 2, A and B). Immunostaining was carried out using specific antibodies against holo-cytochrome *c* reacting with a native protein or apocytochrome *c* reacting with nonnative proteins, including unfolded and denatured proteins. Immunostaining of the cells by the antibody against native cytochrome *c* in absence of heat treatment showed a punctate pattern merging with the mitochondria, visualized with MitoTracker red (Figure 2A). A similar staining pattern was observed when cells were exposed to heat stress at 37°C for 1 h (Figure 2A), although the distribution of mitochondria was significantly altered by stress. These results indicate that holo-cytochrome *c* is not detectably released into the cytosol and remains mainly associated with mitochondria in heat-treated S2 cells. In contrast, the antibody against denatured cytochrome *c* (apocytochrome *c*) did not produce any detectable staining in cells without heat treatment (Figure 2B). This observation is consistent with the previous report that apocytochrome *c* is readily transported into the mitochondria and is hard to detect in the cytosol under normal conditions (Martin and Fearnhead, 2002). Under heat stress conditions, however, there was obvious staining of denatured cytochrome *c* (apocytochrome *c*), which did not merge with Mito-Tracker Red,

suggesting the accumulation of apocytochrome *c* in the cytosol and little colocalization with the mitochondria (Figure 2B). Cell fractionation experiments confirmed that, in heat-treated cells, a significant amount of cytochrome *c* is present in the cytosol, along with a decrease in the concentration of cytochrome *c* in the mitochondria, whereas we found only little cytochrome *c* in the cytosolic fractions of untreated cells (Figure 2C). The heat-induced accumulation of immunoreactive denatured cytochrome *c* and/or apocytochrome *c* in the cytosol may not be derived from the release of cytochrome *c* from mitochondria in the cells undergoing apoptosis, because heat stress did not induce apoptosis and change cell viability in S2 cells (Figure 2D). The absence of release of cytochrome *c* from the mitochondria during apoptosis in *Drosophila* cells, as opposed to its release from mammalian cells during apoptosis, is supported by previous studies (Varkey *et al.*, 1999; Dorstyn *et al.*, 2002). Furthermore, cycloheximide treatment of the cells caused the disappearance of apocytochrome *c* in the cytosol even under heat stress conditions (Figure 2C, right), suggesting that accumulated apocytochrome *c* in the cytosol after heat stress is mainly derived from newly synthesized apocytochrome *c* and that a major contingent of apocytochrome *c* is retained in the cytosol without being imported into the mitochondria under heat-stressed conditions. Holo-cytochrome *c* consists predominantly of α -helices and is converted in vitro by heat >65°C from its native form to insoluble aggregates that are rich in β -sheets (Dong *et al.*, 2000). In contrast, apocytochrome *c*, an unfolded precursor protein of cytochrome *c*

Figure 3. Decrease in 14-3-3 ζ by RNAi causes retardation of the dissolution of aggregated apocytochrome *c* in cells exposed to heat stress. (A) S2 cells were either untreated (control) or treated with dsRNA against 14-3-3 ζ or Hsp83 for 4 d and then heated to 37°C for 1 h. Insoluble (left) and soluble (right) protein fractions were prepared after subsequent incubation at 27°C for various times and immunoblotted with anti-cytochrome *c* antibody. NHS, extracts of cells without heat treatment. Localization of apocytochrome *c* in 14-3-3 ζ - or Hsp83-reduced cells demonstrated by immunostaining. S2 cells treated with 14-3-3 ζ or Hsp83 dsRNA were incubated at 37°C for 1 h. At the indicated times of a recovery period at 27°C, cells were stained for apocytochrome *c* and 14-3-3 ζ (B) or Hsp83 (C). In B and C, the arrowheads indicate 14-3-3 ζ and Hsp83 knockdown cells and the arrows indicate 14-3-3 ζ - and Hsp83-expressing cells.

without heme, is readily aggregated by heat stress at neutral pH (Fisher *et al.*, 1973; Privalov and Makhatadze, 1990). To characterize the basic apocytochrome *c* ($pI = 9.6$) accumulated in cells after heat treatment, we performed acidic native-PAGE, which is suitable to separate basic proteins. Acidic native-PAGE followed by immunoblotting using an apocytochrome *c*-specific antibody revealed that high-molecular-weight apocytochrome *c*, indicating its oligomerization, was detected in untreated S2 cells but its accumulation was sharply enhanced by heat treatment (Figure 2E). These observations suggest that apocytochrome *c* forms aggregates in the cytosol of heat-treated cells.

RNAi of 14-3-3 ζ Causes Retardation of the Dissolution of Aggregated Apocytochrome *c* in Cells Exposed to Heat Stress

We next analyzed the role of 14-3-3 ζ in the aggregation of apocytochrome *c* in heat-treated cells. To determine the intracellular thermolability of apocytochrome *c*, S2 cells were heated at 37°C for 1 h and then transferred into optimal culture conditions at 27°C for various times, followed by separation of the cell extracts into soluble and insoluble fractions according to the protocol described previously (Mogk *et al.*, 1999). Significant amounts of insoluble cytochrome *c* were observed immediately after heat treatment, which were quickly converted into the soluble form within 1 h under culture conditions of recovery at 27°C (Figure 3A). When we reduced the endogenous 14-3-3 ζ levels from S2 cells by RNAi (Figure 1C), the dissolution of insoluble cytochrome *c* during the recovery phase was significantly suppressed, whereas that of insoluble cytochrome *c* was not suppressed by the reduction of cellular Hsp83 levels (Figure 3A, middle and bottom, respectively).

We then analyzed, by immunofluorescence, the effects of reduction of 14-3-3 ζ by RNAi on the accumulated apocytochrome *c* in the heat-treated cells. Immediately after heat

treatment for 1 h at 37°C (0 h of recovery), there was no big difference in the amount of apocytochrome *c* accumulated in the cytosol between cells expressing normal (arrow) versus those expressing reduced (arrowheads) levels of 14-3-3 ζ (Figure 3B). However, after recovery of the S2 cells for 3 h at 27°C, little apocytochrome *c* was detected in the cells expressing 14-3-3 ζ at normal levels, whereas accumulated apocytochrome *c* was retained in the cytosol of 14-3-3 ζ knockdown cells (Figure 3B). Because nearly the same amount of (total) soluble cytochrome *c* was detected in the cells after the recovery for 3 h at 27°C as that detected in heat-untreated cells (Figure 3A), the absence of staining of apocytochrome *c* in the cells expressing 14-3-3 ζ after recovery seemed attributable to the mitochondrial import of resubilized apocytochrome *c* rather than to its degradation. Treatment of the cells with Hsp83 dsRNA did not influence the disappearance of apocytochrome *c* during the recovery phase (Figure 3C). This suggests that 14-3-3 ζ plays a crucial role in the dissolution of insoluble apocytochrome *c* and in the prevention of its accumulation under conditions of heat stress in vivo.

14-3-3 ζ Converts Heat-generated Insoluble Apocytochrome *c* Aggregates into a Soluble Form

To examine the interaction of 14-3-3 ζ with insoluble aggregates of heat-generated cytochrome *c*, we immunoprecipitated by using each specific antibody holo- and apocytochrome *c* in soluble fractions and aggregates solubilized with RIPA buffer from cells before and after heat stress. Western blots analysis of the immunoprecipitates with the anti-14-3-3 ζ mAb revealed the coimmunoprecipitation of 14-3-3 ζ and apocytochrome *c* in insoluble aggregates after but not before heat treatment (Figure 4A, top, lanes 1 and 2). In contrast, 14-3-3 ζ was barely detectable in the immunoprecipitates of holocytochrome *c*, whether the cells had been exposed to heat stress or not (Figure 4A, top, lanes 4 and 5).

Figure 4. Resolubilization of heat-generated insoluble apocytochrome *c* by recombinant 14-3-3 ζ . (A) Coimmunoprecipitation (IP) of 14-3-3 ζ with apocytochrome *c* in insoluble fractions of heat-treated S2 cells. Soluble and insoluble fractions of S2 cells treated with or without heat stress were prepared as described in Figure 3. Insoluble fractions were then solubilized with RIPA buffer. The immunoprecipitates with (apo, holo) anti-cytochrome *c* and normal mouse IgG (cont) antibodies were followed by immunoblotting with the antibodies against 14-3-3 ζ , ϵ and denatured cytochrome *c* antibody. (B) Silver-stained SDS-PAGE of recombinant 14-3-3 ζ (left) and immunoblot analysis of recombinant DnaK and 14-3-3 ζ using a DnaK-specific antibody (right). C and D, effect of exogenous 14-3-3 ζ on the dissolution of aggregated apocytochrome *c*. (C) After incubation of S2 cells treated with dsRNA against 14-3-3 ζ at 37°C for 1 h, exogenous 14-3-3 ζ (middle) or Hsp90 (bottom) was added to the insoluble fraction, followed by treatment at 27°C for up to 2 h. The mixtures were subsequently treated with PK for 10 min, and the susceptibility of apocytochrome *c* to PK was evaluated by immunoblotting with anti-apocytochrome *c* antibody. An insoluble fraction was similarly treated with PK in absence of exogenous proteins and immunoblotted (top). Quantification of the band intensities in arbitrary unit (arb-u) is shown in the bottom panels. (D) 14-3-3 ζ or Hsp90 was added to the same insoluble fraction of C in absence or in presence of ATP and incubated at 27°C for 4 h. Aggregated and soluble proteins were separated by centrifugation, and the insoluble (top) or soluble (bottom) apocytochrome *c* was detected by immunoblotting. Bottom, quantification of the band intensities of insoluble apocytochrome *c*. The panels are representative of three separate experiments.

14-3-3 ζ was hardly detected in the immunoprecipitates of soluble fractions with anti-cytochrome *c* (holo or apo) antibodies, regardless of the exposure of the cells to heat stress (Figure 4A). When the immunoprecipitates of both apo- and holocytochrome *c* were probed with antibody against 14-3-3 ϵ , no significant coimmunoprecipitation of 14-3-3 ϵ with cytochrome *c* was detected, even after incubation of the S2 cells at 37°C for 1 h (Figure 4A). The amounts of holo- and apocytochrome *c* immunoprecipitates of insoluble and soluble fractions are displayed in the bottom panel. These results suggest that 14-3-3 ζ is incorporated into the aggre-

gates containing apocytochrome *c* or colocalizes with them to interact with the aggregated apocytochrome *c* in S2 cells exposed to heat stress. We then examined the dissolution by exogenous recombinant 14-3-3 ζ of insoluble apocytochrome *c* generated in S2 cells treated with 14-3-3 ζ RNAi and heat stress. The isolated insoluble materials from the cells were incubated at 27°C for various times in absence or presence of exogenous recombinant 14-3-3 ζ , after confirmation that the preparation was free from DnaK (Figure 4B). The samples were then subjected to PK digestion on ice for 10 min, to analyze the conformational changes in apocytochrome *c*

coupled with proteolytic susceptibility. Insoluble apocytochrome *c* without 14-3-3 ζ supplementation was poorly digested (Figure 4C), indicating that insoluble apocytochrome *c* is in a highly aggregated state. In contrast, in presence of 14-3-3 ζ , >80% of insoluble apocytochrome *c* became susceptible to PK digestion after incubation for 1 and 2 h (Figure 4C). Hsp90 supplementation, however, had no significant effect on the susceptibility of insoluble apocytochrome *c* to PK digestion, as was the case with the insoluble material in absence of 14-3-3 ζ (Figure 4C). These findings suggest that 14-3-3 ζ interacts with aggregated apocytochrome *c* and induces conformational changes resulting in its resolubilization. Furthermore, insoluble apocytochrome *c* separated by centrifugation was incubated for 4 h at 27°C with or without recombinant 14-3-3 ζ and ATP and then analyzed by SDS-PAGE. Immunoblot analysis using a cytochrome *c* antibody revealed that exogenous 14-3-3 ζ and ATP solubilized ~70% of aggregated apocytochrome *c* in 14-3-3 ζ knockdown cells exposed to heat stress, whereas in absence of ATP or 14-3-3 ζ , little resolubilization of aggregated apocytochrome *c* was observed. The addition of both Hsp90 and ATP also, but in less extent than 14-3-3 ζ and ATP, solubilized ~30% of aggregated apocytochrome *c* (Figure 4D).

14-3-3 ζ Prevents Thermal Aggregation of Proteins and Mediates Their Solubilization In Vitro

To confirm the chaperone activity of 14-3-3 ζ , we examined its protection of purified apocytochrome *c* against heat-dependent aggregation in vitro. Because apocytochrome *c* forms aggregates at neutral pH in vitro, apocytochrome *c* was prepared from holocytochrome *c* in an acidic aqueous solution, pH 4.0–5.0 (Fisher *et al.*, 1973; Hamada *et al.*, 1993). We then incubated soluble apocytochrome *c* at 25°C in reaction buffer, pH 5.0 or 7.5, and monitored its pH-dependent aggregation by light scattering. At neutral pH, a slight, although noticeable aggregation of apocytochrome *c* was observed compared with that observed under acidic conditions (Figure 5A). An increase in temperature from 25 to 48°C at neutral pH caused further aggregation of apocytochrome *c*, whereas at pH 5.0, it remained soluble up to 48°C. We tested the preventive effects of 14-3-3 ζ against the aggregation of apocytochrome *c* at pH 7.5 during heat stress. The addition of 14-3-3 ζ before incubation at 48°C effectively suppressed the formation of apocytochrome *c* aggregates (Figure 5A). Hsp90 also inhibited the thermal aggregation, although the concentration of Hsp90 required for an equivalent degree of inhibition was twofold greater than that of 14-3-3 ζ . Bovine serum albumin as a control protein instead of the chaperones did not prevent the aggregation of apocytochrome *c*. We further investigated whether 14-3-3 ζ is able to resolubilize the heat-aggregated apocytochrome *c*. Apocytochrome *c* (400 nM) aggregated at 48°C was incubated with or without 14-3-3 ζ or Hsp90 in the refolding buffer containing ATP at 25°C, and the disaggregation was monitored by light scattering after an initial unstable phase for 5 min by the addition of proteins. The aggregates remained insoluble in the absence of chaperones (Figure 5B). In contrast, 200 nM 14-3-3 ζ reduced turbidity by ~30% after incubation at 25°C for 30 min (Figure 5C), whereas only ~10% of disaggregation was observed with 200 nM of Hsp90 (Figure 5E). The decrease in aggregate turbidity was more striking when the 14-3-3 ζ concentration was increased from 200 to 400 nM (1:1 14-3-3 ζ to apocytochrome *c* ratio) in the refolding buffer (Figure 5D). These observations indicate that 14-3-3 ζ dissolves heat-aggregated apocytochrome *c* and that its dissolving efficiency was better than that of

Figure 5. Anti- and disaggregation activities of the 14-3-3 protein on apocytochrome *c*. (A) Inhibition of the thermal aggregation of apocytochrome *c* by 14-3-3 ζ . The pH-dependent aggregation of apocytochrome *c* was determined by adding 400 nM apocytochrome *c* to the reaction buffer at 25°C pH 5.0 ($\diamond$) or 7.5 ($\blacksquare$). Thermal aggregation of apocytochrome *c* at 48°C was assessed in absence ($\bullet$) versus presence of 100 nM ($\blacktriangle$) or 200 nM of 14-3-3 ζ ($\square$), or 200 nM Hsp90 ($\circ$), or 200 nM bovine serum albumin ($\triangle$) in reaction buffer at pH 7.5. At the times indicated, the samples were monitored for apparent absorbance at 320 nm, indicative of light scattering because of apocytochrome *c* aggregation. Relative scattering is expressed in arbitrary units. (B–E) Chaperone-dependent disaggregation of heat-aggregated apocytochrome *c*. Apocytochrome *c* (400 nM) incubated at 48°C for 10 min was added to the refolding buffer containing ATP in a thermostated quartz cuvette. Disaggregation of apocytochrome *c* was determined by following the decrease in the light scattering signal at 25°C either in absence (B) or in presence of 200 nM (C) or 400 nM 14-3-3 ζ (D), or 200 nM hsp90 (E). Decrease in turbidity was calculated as a percentage of the initial and highest turbidity generated for ~5 min after addition of aggregated apocytochrome *c* and the chaperones.

Hsp90. To verify the disaggregating effects of 14-3-3 ζ , we examine its effect on the thermal aggregation of CS, a model substrate to analyze chaperone activity. Soluble CS became aggregated with an increase in its turbidity during incubation at 43°C. The rate and extent of CS aggregation were inhibited in a dose-dependent manner by the addition of 14-3-3 ζ (Figure 6A). The resolubilization effects of 14-3-3 ζ toward heat-aggregated CS was analyzed by incubation at 25°C for 4 h in refolding buffer with or without 14-3-3 ζ and ATP. After incubation, CS aggregates were isolated by centrifugation and subjected to SDS-PAGE. An ~60% reduction in insoluble CS was observed in presence of both 14-3-3 ζ and ATP (Figure 6B), whereas in absence of ATP, no resolubilization of CS aggregates by 14-3-3 ζ was observed.

14-3-3 ζ Mediates the Reactivation of Heat-aggregated Citrate Synthase with Other Chaperones

Protein disaggregation activity, a unique property of heat-inducible members of the Hsp100 family conserved in yeast and eubacteria, as Hsp104 and ClpB, has been described as a key factor in the chaperone-assisted reactivation of heat-

Figure 6. Inhibition and disaggregation of heat aggregation of CS by 14-3-3 ζ . (A) 14-3-3 ζ suppresses the aggregation of CS. Native CS was diluted to a final concentration of 200 nM in the reaction buffer at 43°C in absence (■) or presence of 100 nM (▲), 150 nM (◆), or 200 nM (●) 14-3-3 ζ . Light scattering by CS was monitored at 320 nm. (B) 14-3-3 ζ disaggregates thermal-aggregated CS. CS heated at 25 or 43°C for 5 min was added to the refolding buffer in absence or presence of 14-3-3 ζ and then incubated at 25°C for additional 4 h. Insoluble CS was separated by centrifugation for 20 min at 20,000 $\times$ g and detected by silver staining. Quantification of the band intensities in arbitrary unit (arb-u) is shown in the bottom panel.

denatured proteins (Glover and Lindquist, 1998; Mogk *et al.*, 1999). Hsp104 (ClpB) mediates the reactivation of heat-denatured proteins in conjunction with Hsp70 (DnaK) and Hsp40 (DnaJ/GrpE) by promoting their resolubilization from an aggregated form, whereas Hsp104 (ClpB) is insufficient by itself for their reactivation. Therefore, we next investigated the effect of 14-3-3 ζ on the reactivation of heat-aggregated CS. 14-3-3 ζ by itself did not show any reactivation activity for heat-aggregated CS in comparison with a

Figure 7. 14-3-3 ζ -mediated reactivation of heat-aggregated CS in cooperation with Hsp70/Hsp40. CS (150 nM) was incubated at 43°C for 10 min in 40 mM HEPES-KOH buffer, at pH 7.5, for aggregation. Reactivation of the heat-aggregated CS was initiated in refolding buffer containing 2 mM ATP at 25°C in the presence of 14-3-3 ζ alone (300 nM; ○); Hsp70 and Hsp40 (300 and 150 nM, respectively; □); 14-3-3 ζ , Hsp70, and Hsp40 (300, 300, and 150 nM, respectively; ●); bovine serum albumin, Hsp70 and Hsp40 (300, 300, and 150 nM, respectively; ■), or in absence of chaperones (Δ). CS activity was analyzed at the indicated times, and relative enzyme activity was expressed as a percentage of native CS activity.

spontaneous reactivation of heat-aggregated CS in the refolding buffer at 25°C without a chaperone (Figure 7). Further addition of Hsp70 and Hsp40 to the assay system in the presence of 14-3-3 ζ caused stimulated reactivation of heat-aggregated CS, whereas no stimulation of reactivation by Hsp70/Hsp40 was observed without 14-3-3 ζ in the refolding mixture. These results indicate that not only 14-3-3 ζ but also additional specific chaperones are required for the refolding of aggregated protein substrate. In contrast, no resolubilization of heat-aggregated CS was detected when heat-aggregated CS was incubated with Hsp70, Hsp40 alone, or with Hsp70/Hsp40 (our unpublished data). These data suggest that 14-3-3 ζ in cooperation with Hsp70/Hsp40 reactivates the heat-inactivated CS from insoluble aggregates to soluble renaturalized form in vitro.

DISCUSSION

This study revealed that the expression of the *Drosophila* 14-3-3 ζ , constitutively expressed under nonstressed conditions, is up-regulated by transcriptional activation after exposure to heat stress. The expression of heat-shock proteins is generally regulated transcriptionally by de novo synthesis of hsp mRNAs, posttranscriptionally via an effect on message stability, or at the translation initiation step (Morimoto *et al.*, 1994). The inhibitory effect of actinomycin D on an increase in 14-3-3 ζ expression (Figure 1B) suggests that its induction during heat stress is regulated at the transcriptional level.

In eukaryotic cells, HSF is a stress-inducible transactivator of the heat shock response, which binds to HSE. HSE is generally composed of repeated arrays of the 5-base pairs module [nGAAn], which has a high binding affinity for HSF (Amin *et al.*, 1988; Xiao and Lis, 1988). Like other heat-shock genes which include this 5-base pairs building block within their promoters, the *Drosophila* 14-3-3 ζ gene has a consensus unit [gGAAacGAAC] arranged as contiguous repeats, located ~360 bases upstream from the transcriptional start site. These observations suggest the involvement of *Drosophila* HSF in the synthesis of 14-3-3 ζ in response to heat stress. Moreover, the ChIP assay showed a heat-shock-dependent HSF interaction with the 14-3-3 ζ gene promoter in vivo (Figure 1E), and HSF RNAi markedly suppressed the up-regulation of 14-3-3 ζ under heat stress conditions (Figure 1D). This is the first report of the up-regulation of 14-3-3 ζ mediated by HSF upon exposure to heat stress.

Heat-shock proteins protect other proteins against aggregation to survive during heat stress (Langer *et al.*, 1992; Schroder *et al.*, 1993). In this study, we found a new cellular function of 14-3-3 ζ as a molecular chaperone. Heat stress caused the accumulation of insoluble apocytochrome *c* in the cytosol of S2 cells, consequently reducing the mitochondrial cytochrome *c* (apo and/or holo). When the intracellular 14-3-3 ζ was depleted by RNAi, the dissolution of insoluble apocytochrome *c* during the recovery phase at 27°C, observed in control cells, was markedly suppressed. This dissolution activity of 14-3-3 ζ and its colocalization with the aggregated apocytochrome *c* suggest that 14-3-3 ζ interacts directly with the aggregated apocytochrome *c* in S2 cells exposed to heat stress. In contrast, the transient accumulation of unfolded proteins during heat stress is probably because of limited amounts of molecular chaperons in the extremely crowded macromolecular environment (Glover and Lindquist, 1998).

Another important observation of 14-3-3 ζ in this study was the resolubilization by exogenous 14-3-3 ζ supplementation of the heat-aggregated apocytochrome *c* in cell ex-

tracts and in vitro. In addition, a similar effect of 14-3-3 ζ on heat-aggregated CS was observed, suggesting that it mediates the resolubilization of several protein aggregates under heat-induced pathological conditions. The ability of heat shock proteins to rescue other proteins from aggregates has been reported in a few cases, such as yeast Hsp104 and its prokaryotic homologue ClpB (Parsell *et al.*, 1994; Mogk *et al.*, 1999; Ben-Zvi and Goloubinoff, 2001; Zietkiewicz *et al.*, 2004). Although the fundamental mechanism behind the resolubilization of insoluble proteins is unknown, Glover and Lindquist have proposed that Hsp104 solubilizes the aggregates by pulling apart the oligomeric assemblages of heat-damaged proteins. The observation of an increase in the susceptibility to PK digestion of the heat-aggregated apocytochrome *c* by 14-3-3 ζ supplementation suggests that like Hsp104, 14-3-3 ζ may drive the aggregated apocytochrome *c* into a relaxed state. We also observed that the 14-3-3 ζ -mediated resolubilization of heat-aggregated CS allows the subsequent refolding of CS by Hsp70/Hsp40 in vitro. Neither 14-3-3 ζ nor Hsp70/Hsp40 alone reactivated heat-aggregated CS. The 14-3-3 ζ -mediated refolding of aggregated CS that occurs only in conjunction with other chaperones is noteworthy. One possible explanation is the breaking of large aggregates by 14-3-3 ζ , which sequester the hydrophobic surfaces of substrate, into smaller species that are more accessible to other chaperones, thus facilitating protein refolding. The unique characteristics of the 14-3-3 protein that we have identified may establish its position as a stress tolerance factor that promotes the reactivation of heat-aggregated proteins in the chaperone machinery, forming a so-called "bichaperone network."

In addition, the stress-related chaperone functions of 14-3-3 ζ are clearly distinct from previously described roles played by the mitochondria import stimulation factor, which facilitates the transport of newly synthesized mitochondrial precursors into mitochondria. Therefore, these newly described properties of the 14-3-3 will promote further studies aimed at clarifying the multiple functions of 14-3-3 protein in cells.

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