Hsp40 Couples with the CSP\textalpha Chaperone Complex upon Induction of the Heat Shock Response

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Abstract

In response to a conditioning stress, the expression of a set of molecular chaperones called heat shock proteins is increased. In neurons, stress-induced and constitutively expressed molecular chaperones protect against damage induced by ischemia and neurodegenerative diseases, however the molecular basis of this protection is not known. Here we have investigated the crosstalk between stress-induced chaperones and cysteine string protein (CSP\textalpha). CSP\textalpha is a constitutively expressed synaptic vesicle protein bearing a J domain and a cysteine rich “string” region that has been implicated in the long term functional integrity of synaptic transmission and the defense against neurodegeneration. We have shown previously that the CSP\textalpha chaperone complex increases isoproterenol-mediated signaling by stimulating GDP/GTP exchange of G\textalpha. In this report we demonstrate that in response to heat shock or treatment with the Hsp90 inhibitor geldanamycin, the J protein Hsp40 becomes a major component of the CSP\textalpha complex. Association of Hsp40 with CSP\textalpha decreases CSP\textalpha-CSP\textalpha dimerization and enhances the CSP\textalpha-induced increase in steady state GTP hydrolysis of G\textalpha. This newly identified CSP\textalpha-Hsp40 association reveals a previously undescribed coupling of J proteins. In view of the crucial importance of stress-induced chaperones in the protection against cell death, we data attribute a role for Hsp40 crosstalk with CSP\textalpha in neuroprotection.

Introduction

In response to a range of stressful stimuli including hyperthermia and ischemia, an ancient evolutionarily conserved cellular program called the heat shock response is activated and the expression of several chaperones is induced to enhance cell survival to subsequent insults. The heat shock response also involves the translocation of several chaperones [1,2]. Although the mechanistic basis of chaperone cytoprotection is not yet understood, the chaperone anti-apoptotic activity is thought to be due to the ability of chaperones to rid the cell of misfolded proteins. In addition to the stress-induced chaperones, many molecular chaperones are expressed constitutively and are widely held to have basic and indispensable functions in maintaining protein conformation. Constitutive and stress-induced chaperones maintain an important balance in the cell between protein refolding and protein elimination, however, while transient up-regulation of molecular chaperones is critical for cell survival, chronic up-regulation of chaperones (eg Hsp70) correlates with poor prognosis in some types of cancer [3]. Neurons are thought to be especially vulnerable to the risk of protein misfolding due to the conformational flexibility required of the cellular machinery underlying synaptic transmission. Furthermore, post-mitotic cells, such as neurons, cannot dilute aggregated proteins through cell division, leading to the potential accumulation of misfolded proteins. Huntington’s, Alzheimer’s, Parkinson’s and Prion diseases are caused by defects in protein folding, underlining the biological importance of the problem of aberrant protein folding in neurons. In experimental models, molecular chaperones, either constitutive or stress-induced, are inhibitors of neurodegeneration (reviewed: [4–6]). Pharmacological modulators that directly regulate chaperone and stress-induced chaperone activity have been identified, emphasizing the potential of the heat shock response as a pharmaceutical target (Reviewed: [7]).

Cysteine string protein (CSP\textalpha) is a synaptic vesicle protein bearing a signature J domain and a cysteine rich string region that is implicated in the defense against neurodegeneration. The cytosolic proteins Hsc70 (heat shock cognate protein of 70 kDa), SGT (small glutamine rich tetratricopeptide repeat domain protein) and HIP (Hsc70 interacting protein) form a complex with CSP\textalpha which is tethered to the synaptic vesicle. Assembly of the chaperone components is thought to allow for localized activation of Hsc70, a cytosolic ATPase that couples energy from ATP hydrolysis to conformational work on target proteins [8–11]. The assembly of the CSP\textalpha chaperone complex is nucleotide sensitive, emphasizing the dynamic nature of the complex [8,10,12]. Why a specialized synaptic vesicle chaperone system evolved remains a mystery.

The existence of the heat shock response raises several questions. How do stress-induced chaperones protect synaptic transmission and prevent neurodegeneration? Is there crosstalk...
between stress-induced chaperones and constitutively expressed chaperones or do these chaperone machines have separate non-overlapping cellular tasks? As a first step toward testing the hypothesis that stress-induced chaperones are coupled to the CSPx chaperone system, we evaluated the components of the CSPx complex during transient expression of stress chaperones. CSPx expression levels do not increase in response to heat shock or geldanamycin treatment in neural cell lines. The previously reported 70 kDa CSPx dimer \[13,14\] was reduced following heat shock. Surprisingly, our findings demonstrate that the stress-induced J protein Hsp40 becomes a major component of the CSPx multimeric complex after heat shock. Following heat shock, Hsp40 expression is increased and Hsp40 localizes to the plasma membrane. Geldanamycin, like heat shock, triggers the assembly of Hsp40 with the CSPx multimeric complex. In \textit{vivo}, the stimulatory effect of CSPx on the steady-state hydrolysis of GTP by G\textsubscript{x} is enhanced in the presence of Hsp40. Furthermore, transient transfection of CSPx or induction of the heat shock response increased isoproterenol-stimulated phosphorylation of synapsin.

Our results suggest that the transient assembly of Hsp40 with the CSPx complex is important in the maintenance of synaptic function in the face of environmental stress, and emphasize the complexity and functional elegance of the J protein chaperone machines.

**Results**

**CSPx expression is not altered in response to heat shock**

To begin to test the role of CSPx in the heat shock response, the expression of CSPx and Hsp70 was examined in CAD mouse neuroblastoma cells before and after 40 min of conditioning heat shock at 42°C. CSPx is constitutively expressed in brain and exocrine/endocrine secretory tissues, however, its expression has been reported to be influenced by antidepressants \[15–17\], amphetamines \[18\] and diabetes \[19\]. The cellular mechanisms that underlie changes in CSPx expression are currently unknown. The cDNA clone for rat CSPx (594 bp open reading frame) contains 181 nucleotides of 5’ untranslated region, 1.2 kb of 3’ untranslated region and encodes a 35 kDa protein with extensive lipid modification \[13\]. Figure 1 shows that CSPx levels are not increased by heat shock. As expected, Hsp70 and Hsp40 were induced by heat shock and their induction was blocked by pretreatment with quercetin, an inhibitor of the heat shock response. Quercetin did not alter CSPx expression.

We then evaluated the time course of expression of select chaperones in response to heat shock. Following a conditioning stress, Hsp70 is rapidly expressed in CAD, LAN1, and PC12 cell lines, consistent with Hsp70 induction in neurons \[1\]. Figure 2 demonstrates that Hsp70 protein expression is clearly increased \(~3\) hours after heat shock. Although Hsp70 is detected in LAN1 cells prior to the conditioning stress, the levels of Hsp70 expression were still observed to increase in response to heat shock. The time course of Hsp70 expression showed slower onset in differentiated compared to undifferentiated CAD cells, however robust Hsp70 expression was observed at \(~6\) hours in differentiated CAD cells. Figure 2 shows that Hsp25/27 expression also increased in CAD, LAN1, PC12 cell lines \(~5\) hours after a conditioning heat shock. Although Hsp25/27 was expressed in unstimulated LAN1 and PC12 cells, further increases were observed following a conditioning heat shock. Hsp40 levels increased \(~3\) hours after a conditioning heat shock in CAD and LAN1 cells, however, no increase was seen in PC12 cells which already had high control levels of Hsp40 pre-heat shock. In contrast to Hsp70, Hsp40 and Hsp25/27, the expression of actin was not altered in response to heat shock demonstrating the specificity of the cellular response to a conditioning stress. Following heat shock CSPx levels are not altered in CAD cells (Figure 1), however in PC12 and LAN1 cells reduced CSPx levels were observed (Figure 2). These data are consistent with those found \textit{in vivo} where the severity of the stress required to trigger the heat shock response is reported to vary among neural populations \[6\].

**Hsp70 and Hsp25/27 expression is variable among neural cell lines**

Next we directly compared non-heat shocked (control) CAD, differentiated CAD, PC12, differentiated PC12, LAN1 and HEK cells for CSPx expression. Figure 3A shows that CSPx was present in all cell lines except HEK cells. The cell lines were then evaluated for basal (control) levels of heat shock chaperones including Hsp70, Hsp25/27 (heat shock protein 25/27) and Hsp40. Hsp70 was not detectable in non-heat shocked (control) CAD, differentiated CAD or PC12 cells, but was present at high

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**Figure 1. Effect of heat shock on native CSPx expression.** (A) CAD cells were treated with or without 200 \textmu M quercetin for 24 hours followed by heat shock for 40 min at 42°C and allowed to recover for 5 hours. 30 \mu g of protein was resolved by SDS-PAGE. CSPx, Hsp70 and Hsp40 were detected by Western analysis. (B) Quantification of CSPx. Data were derived from a total of 8 separate experiments. doi:10.1371/journal.pone.0004595.g001
levels in LAN1 and HEK cells and detectable in differentiated PC12 cells. Notably, some tumor cells have been reported to constitutively express high levels of the anti-apoptotic chaperone Hsp70. Furthermore, high Hsp70 levels have been reported to correlate with poor prognosis in some types of cancer [3,7]. In contrast, Hsp25/Hsp27 was absent from CAD cells but detectable in HEK cells and abundant in LAN1 and PC12 cells. Hsp25 and Hsp27 are homologous proteins. Hsp25 is present in rat and mouse, while Hsp27 is present in humans. Anti-Hsp25 antibody does not cross-react with Hsp27 and vice versa. Actin is shown as a loading control (Figure 3, panel 5). The basal expression of select chaperones in unstressed rat brain is shown for comparison in Figure 3B. Hsp40 and Hsc70 are abundant in both the cytosolic (S) and membrane particulate (M) fractions of rat brain while the stress-inducible Hsp70 was not detected. Hsp25 was detected in the cytosolic fraction. Taken together, these observations indicate that while CSPγ was present in all neural cell lines as expected, the background expression of stress-induced chaperones varied extensively between the cell lines.

The CSPγ dimer is reduced by heat shock and increased by quercetin treatment

Although CSPγ is present in neural cell lines, its expression is ~10 fold lower (10±1.7, n = 3, data not shown) than that found in rat brain homogenates. To conduct a more detailed analysis of the CSPγ chaperone complex, CSPγ was examined in CAD mouse neuroblastoma cells transiently transfected with CSPγ in order to bring CSPγ levels up to those found in adult rat brain. After transfection a 70 kDa CSPγ immunoreactive band was observed (Figure 4). 70 kDa CSPγ dimers have previously been reported in rat brain [13,20], rat hippocampus [12], rat pancreas [13], PC12 cells transiently expressing CSPγ [14,21], and HEK293 cells transiently expressing CSPγ [22]. The cellular role of the CSPγ dimer is not known. Figure 4 shows that in CAD cells transfected with CSPγ the 70 kDa CSPγ dimer is stable, SDS-resistant and maintained after incubation in sample buffer at 80°C for 10 min. Figure 4 clearly demonstrates that following 40 minutes of conditioning heat shock at 42°C, there is a decline in the CSPγ dimer detected. In contrast, quercetin increases the CSPγ-CSPγ complex in control and heat shocked cells (Figure 4). The upper panel in Figure 4 is an overexposure of the 70 kDa CSPγ dimer demonstrating its presence in control but not heat shocked CAD cells. In transfected (Figure 4) but not untransfected (Figure 1) CAD cells, quercetin was observed to increase expression levels of the CSPγ monomer. Taken together, these data show that the extremely stable CSPγ dimer is regulated by quercetin as well as by a conditioning heat shock.

Hsp40 is a major component of the CSPγ complex after a conditioning heat shock

Two possible scenarios could mediate the anti-apoptotic activity of stress chaperones. It is possible that (1) stress inducible chaperones have evolved to perform the same function as constitutively expressed chaperones or that (2) they carry out specialized functions to specifically cope with physiological stress.

Figure 2. Time course of heat shock in CAD, LAN1, PC12 and differentiated CAD cells. (A) CAD, (B) LAN1 and (C) PC12 cells were heat shocked for 30 min at 42°C and allowed to recover for indicated times. (-) no heat shock. (D) CAD cells were differentiated by 36 hours serum starvation. 20 μg of protein was resolved by SDS-PAGE. Hsp70, Hsp25, Hsp27, Hsp40, Hsp90, CSPγ and actin were detected by Western analysis. Data are representative of 4 separate experiments. doi:10.1371/journal.pone.0004595.g002

Hsp040/CSPγ Chaperone Complex
In order to distinguish between these two possibilities, we examined the association between CSP\(\alpha\) and heat shock proteins. Glutathione-S-transferase (GST) fusion proteins consisting of CSP\(\alpha_{1-112}\) or CSP\(\alpha_{1-198}\) (full length) were coupled to glutathione agarose beads and used in an \textit{in vitro} binding assay. The beads were incubated with cell homogenate, washed, and bound proteins were eluted and evaluated by Western blot. We have previously shown that CSP\(\alpha_{1-112}\) contains two binding sites for G proteins and has guanine nucleotide exchange (GEF) activity for G\(\alpha_s\) [11]. Recombinant full length CSP\(\alpha_{1-198}\) also contains both binding sites for G proteins, but only has GEF activity for G\(\alpha_s\) in the presence of Hsc70 and SGT (small glutamine-rich tetrapeptide repeat domain protein). Specifically, we have shown that CSP\(\alpha\) regulates heterotrimeric GTP binding proteins (G proteins) by preferentially targeting the inactive GDP-bound form of G\(\alpha_s\) and promoting GDP/GTP exchange which increases cAMP levels and downstream phosphorylation. \textit{In vitro}, Hsc70 and SGT trigger a switch in CSP\(\alpha\) from an inactive GEF to an active GEF. Therefore we examined both CSP\(\alpha_{1-198}\) (requires Hsc70/SGT activation) and CSP\(\alpha_{1-112}\) (active GEF) for their possible associations with stress-induced chaperones.

Chaperones homologous to those found in the CSP\(\alpha\) complex are induced following a preconditioning heat shock (e.g., Hsp70 (70 kDa heat shock protein) and Hsp40 (40 kDa heat shock protein)), Hsc70 and Hsp70 both associate with the J domain of CSP\(\alpha\) [8,9,20]. Despite the robust induction of Hsp70 expression

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**Figure 3. CSP\(\alpha\) expression in CAD, PC12, LAN1, HEK cells.** CAD cells were differentiated by serum withdrawal. PC12 cells were differentiated by NGF (50 ng/ml 9 days). (A) 25 \(\mu\)g of total cell homogenate. (B) Basal chaperone expression levels in rat brain supernatant (S) or rat brain membranes (M) were resolved by SDS-PAGE. CSP\(\alpha\), Hsp70, Hsp27, Hsp25 and actin (loading control) were detected by Western analysis. Data are representative of 4 separate experiments.
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by heat shock and its significant homology (85%) with Hsc70 [23], a CSPz/Hsc70 complex was favored over a CSPz/Hsp70 complex in CAD cell homogenates (Figure 5). After a conditioning heat shock, Hsp70 was induced and associated weakly with the GST-CSPz1-112 and CSPz1-198 complex. These data confirm the relatively weaker CSPz association and ATPase activation of Hsp70 compared to Hsc70 utilizing yeast two hybrid and ATPase assay techniques [9,20]. The ATPase Hsp90 weakly associated with the CSPz complex. Like Hsc70, Hsp70 association was increased in the presence of ATP. In contrast, Hsp25 was not found to associate with the CSPz complex either before or after heat shock.

If Hsp40 and CSPz have evolved to perform separate functions, then it is possible that induction of Hsp40 will reduce the assembly of CSPz with Hsc70. Given the homology between the J domains of CSPz and Hsp40, one might expect elevated levels of Hsp40 to disrupt the CSPz/Hsc70 complex and favor a Hsp40/Hsc70 complex by competing with CSPz for association with Hsc70 and Hsp70. To our surprise, however, Hsp40 was found to associate robustly with both GST-CSPz1-112 and CSPz1-198 after heat shock. Figure 5 shows that Hsp40 association with CSPz1-198 was nucleotide independent, however association of Hsp40 with CSPz1-112 was greater in the presence of either ATP or GDP. These data indicate that the components of the CSPz chaperone complex are altered in response to a conditioning stress to include the cytosolic stress-induced J protein Hsp40.

While these data are consistent with a direct interaction between Hsp40 and CSPz, it does not permit us to rule out the possibility that CSPz/Hsp40 interact indirectly. To investigate this possibility, we examined the ability of immobilized CSPz fusion proteins to interact with soluble Hsp40. As shown in Figure 6A, immobilized GST-CSPz1-112 and GST-CSPz1-198 were able to bind purified, soluble Hsp40 in a pull down assay, indicating that there is indeed a direct interaction between CSPz and Hsp40.

To further understand the structural requirements for assembly of Hsp40 with the CSPz complex, Hsp40 deletion and point mutants were constructed and the regions of Hsp40 required for its binding to CSPz were determined. In each assay, an equal amount of fusion protein was immobilized to sepharose beads as confirmed by Ponceau S staining. The presence of Hsp40 was analyzed by Western blotting. Hsp40 and CSPz belong to a large and diverse protein family [24]. Each member has a conserved J domain that functions to stimulate Hsc70/Hsp70 ATPase (e.g. CSPz stimulates Hsc70 ATPase [8]). There is no functional one to one correspondence between members of the J protein family and members of the Hsp70 family. J domains are a ~70 amino acid region of homology comprised of four α helices with a highly conserved tripeptide of histidine, proline and aspartic acid (HPD motif) located between helices II and III. The structures of the J domain of CSPz (mouse) and Hsp40 (human) have been determined from nuclear magnetic resonance studies [25]. Figure S1 shows the comparison of the amino acid sequence between rat CSPz and Hsp40. The J domain (magenta) and cysteine string region (red) of CSPz and the J domain and the DnaJ C-terminal domain (cyan) of Hsp40 are indicated. CSPz and Hsp40 show only 16% identity over their entire amino acid sequences, but 51% identity within the J domains. Secondary structure predictions for helix 1 show a weak “β sheet” (the probability is 0.4 sheet compared to 0.3 helix; 1 is high, 0 is low) for position 5–8 a.a. of Hsp40_Rat and 17–20 a.a. of CSPz_Rat, while predictions are stronger for helices 2, 3 and 4 (probability is 1, 1 and 0.7 respectively). Mutation of the highly conserved HPD tripeptide of Hsp40 (Hsp40HPD→AAA) did not abolish binding to CSPz, indicating that CSPz/Hsp40 association is not dependent on this conserved motif. Furthermore, Hsp40_C and Hsp40_Rat were not found to associate with CSPz1-112 or CSPz1-198 (Figure 6B&C). These data therefore define Hsp40 residues 81–340 as important for binding to CSPz.

To understand better the nature of the CSPz/Hsp40 association, we evaluated the distribution of CSPz and Hsp40 in CAD cells transiently expressing myc-CSPz (Figure 7). In control cells (C), CSPz was observed to be concentrated at the cell-cell contact sites (indicated with an arrow) and, in contrast, Hsp40 was broadly expressed. Following heat shock (H.S.), we found CSPz and Hsp40 to be present primarily in the plasma membrane however reduced at the cell-cell contacts, indicating that Hsp40 as well as CSPz was relocated. These observations are consistent with our biochemical data (Figures 5&6) demonstrating a CSPz/Hsp40 association following heat shock. Panel 4 clearly demonstrates that quercetin blocked the heat shock general redistribution of Hsp40 to the plasma membrane. Intracellular localization of CSPz and Hsp40 was increased in cells treated with quercetin (Q) as well as quercetin followed by heat shock (Q+HS). To our surprise, Hsp40 but not CSPz localized to cell-cell contacts.
Hsp40/CSPα Chaperone Complex

A

Control | Heat Shock
---|---
H | ATP | GDP | H | ATP | GDP

Hsp40
Hsp70
Hsc70
Hsp25
Hsp90

B

Control | Heat Shock
---|---
H | ATP | GDP | H | ATP | GDP

Hsp40
Hsp70
Hsc70
Hsp25
Hsp90
following the quercetin/heat shock. Plasma membrane co-localization of CSPα and Hsp40 in single differentiated cells is shown in yellow in the right hand panel (panel 5). Taken together, these biochemical and histochemical data demonstrate that following heat shock, cellular levels of Hsp40 are increased and both Hsp40 and CSPα undergo redistribution. These data indicate that changes in the expression levels of Hsp40 as well as changes in the cellular localization of Hsp40 and CSPα facilitate the assembly of Hsp40 with the CSPα chaperone complex.

Hsp40 is a major component of the CSPα complex after Geldanamycin treatment

Next we evaluated a series of agents to see if they altered either CSPα levels or the components of the CSPα complex (Figure S2A). The Hsp90 ATPase inhibitors geldanamycin, 17-AAG and novobiocin robustly induced the expression of Hsp70 in PC12 cells. Lithium ions have been reported to enhance CSPα expression [26] and we have previously shown that CSPα increased the response to the β2 adrenergic agonist isoproterenol [11]. No Hsp70 was detected in PC12 cells treated with isoproterenol, LiCl or quercetin. In contrast to Hsp70 and Hsp25, the expression of actin was not altered in response to geldanamycin or 17AAG demonstrating the specificity of the cellular response to the Hsp90 inhibitors. Following geldanamycin treatment of PC12 cells, Hsp70 expression is detected as early as 5 hours and continues to increase (Figure S2B). To our surprise, geldanamycin did not trigger the expression of Hsp70 but did induce Hsp40 expression without increasing CSPα expression in CAD mouse neuroblastoma cells (Figures S2C&D). The molecular events that underlie the difference between geldanamycin-induced and heat shock-induced Hsp70 expression in CAD cells remains to be established, however, it provided us the opportunity to investigate the CSPα/Hsp40 association in the absence of Hsp70.

We then examined the CSPα complex in geldanamycin treated CAD cells. CSPα1-112 or CSPα1-198 fusion proteins were coupled to glutathione agarose beads and used in an in vitro binding assay. Following geldanamycin treatment, Hsp40-CSPα1-112 and Hsp40-CSPα1-198 complexes were abundant in the presence of ATP (Figure 8), similar to that found after heat shock (Figure 5). Again, Hsp90 was observed to associate weakly with the CSPα complex. Thus the CSPα chaperone complex is dynamic, undergoing changes in components in the post-stress response and in response to Hsp90 inhibitors. Taken together, our results indicate that as the cellular levels of Hsp40 rise in response to a conditioning stress or after treatment with Hsp90 inhibitors, the CSPα complex becomes an Hsp40-CSPα complex.

Hsp40 promotes CSPα’s GEF activity in vitro and synapsin phosphorylation in CAD neuroblastoma cells

Next we evaluated Hsp40’s effect on the steady state hydrolysis of GTP by Gαs. We have previously demonstrated that the stimulation of GTPase activity by Gαs by CSPα requires Hsc70 and SGT1 [11]. Figure 9A shows that Hsp40 enhanced the CSPα-Hsc70-SGT-stimulated increase in GTP hydrolysis by Gαs. GTP hydrolysis by Gαs was not altered in the presence of Hsp40 alone, indicating that Hsp40 does not affect the hydrolysis of GTP by Gαs alone. Furthermore, Hsp40 does not hydrolyze GTP. To gain further insight into the transient association of Hsp40 with the CSPα chaperone complex, we evaluated the activation of cellular mechanisms downstream of G proteins after heat shock. Phosphorylation of synapsin in CAD cells serves as an independent readout of cellular signaling through Gαs. Figure 9B shows that isoproterenol-induced phosphorylation of synapsin in CAD cells was increased after heat shock (3.7 fold) or transfection with myc-tagged CSPα (4.2 fold). This is consistent with previous work showing that expression of CSPα in HEK cells increases isoproterenol-mediated phosphorylation of the transcription factor CREB [27]. These results show that the heat shock response modulates signaling through Gαs pathways.

Discussion

In this study, we demonstrate for the first time that the stress-induced J protein, Hsp40, physically couples with the constitutive CSPα chaperone complex. CSPα is a synaptic vesicle J protein that is essential for periods of extended neurotransmission and is implicated in the defense against neurodegeneration [28–30]. CSPα is constitutively expressed on synaptic vesicles and is thought to tether Hsc70 for conformational work at the synaptic vesicle site. Hsp40 is a cytosolic J protein that is rapidly and transiently induced in response to stress, including, but not exclusive to, heat shock and geldanamycin treatment. Stress-induced expression of several chaperones, including Hsp40, is widely thought to protect cells from the deleterious effects of subsequent stress. Interference with the heat shock response would be expected to have enormous cellular consequences and reduce cell survival. It is fully anticipated that transient expression of stress-induced chaperones in vivo occurs routinely in neurons to cope with the stresses rendered by various insults. Intriguingly, some neurons have a particularly higher threshold for the induction of the heat shock response compared with other neurons [6]. In this study, we provide evidence that Hsp40 specifically and directly associates with the constitutive CSPα synaptic vesicle chaperone complex following either a conditioning heat shock or geldanamycin treatment. Following heat shock, Hsp40 expression is increased and both CSPα and Hsp40 undergo redistribution. The association of Hsp40 with CSPα correlates with reduced CSPα dimerization, translocation of Hsp40 to the plasma membrane and enhanced CSPα-mediated augmentation of steady state GTP hydrolysis. Association with CSPα is mediated via C terminal binding sites of Hsp40 and does not involve the J domain. Since neurotransmitter release relies on complex interactions between multiple cellular components, we speculate that the cross-talk between CSPα chaperone machinery and the stress-induced Hsp40 is important in maintaining functionally competent synapses.

Whether stress-induced chaperones typically perform independent cellular tasks or are coupled to constitutive chaperone machines, such as CSPα, is a current biological question. The notion that Hsp40 contributes to the folding activity of the CSPα

Figure 5. Western analysis showing the association of Hsp40 with CSPα1-112 and CSPα1-198 before and after heat shock. (A&B) CAD cells were heat shocked for 30 minutes at 42°C and allowed to recover for 5 hours. GST fusion proteins of (A) CSPα1-112 and (B) CSPα1-198 were immobilized on glutathione-sepharose and incubated in the presence of 110 µg of control or heat shocked CAD cell homogenate in the presence or absence of 2 mM ATP or GDP. The lane indicated as (H) is 40 µg of cell homogenate loaded directly on the gel. The beads were washed and bound proteins were eluted in sample buffer, fractionated by SDS-PAGE and subjected to Western blot analysis. Hsp70, Hsc70, Hsp25, Hsp40, and Hsp90 were detected by Western analysis.

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Figure 6. Western analysis showing association of CSPα fusion proteins with recombinant Hsp40 and Hsp40_{HPD-AAA}. (A) Immobilized GST-CSPα_{1-112}, GST-CSPα_{1-198}, or GST alone were incubated with full length Hsp40 (i.e. amino acids 1–340), washed and bound Hsp40 was evaluated by Western blot. (B) Immobilized GST-CSPα_{1-198} was incubated with soluble, full length Hsp40_{1-340}, Hsp40_{1-340}_{HPD-AAA}, Hsp40_{1-80} or Hsp40_{1-80}_{HPD-AAA}. Beads were washed, bound proteins eluted in sample buffer and subjected to Western blot analysis with anti-Hsp40 polyclonal (Assay Designs). Lanes are
chaperone complex after a conditioning stress is consistent with the neurodegeneration observed in CSP40 deletion models. Deletion of the CSP40 gene severely impairs central and presynaptic transmission in Drosophila melanogaster [30–32]. The Drosophila CSP40 null mutants exhibit temperature sensitive paralysis and die as larvae or within days of adulthood [30]. Deletion of CSP40 in mice causes blindness followed by progressive motor and sensorial impairment and neurodegeneration with no survival beyond 4 months [29,33,34]. Thus, these results indicate that CSP40 is important for the long term functional integrity of the synaptic machinery. It is possible, given the data presented here that the prevention of presynaptic neurodegeneration by the synaptic vesicle CSP40 chaperone complex involves a transient interaction with the highly conserved stress induced protein Hsp40.

Candidates for the protein substrate(s) of the CSP40/Hsc70 system include: G proteins (heterotrimeric GTP binding proteins) [11,12,35], voltage sensitive calcium channels [36,37] and SNAREs (soluble N ethylmaleimide-sensitive factor attachment protein receptors) [28,38,39]. Furthermore, CSP40 has been shown to be critical for the normal calcium sensitivity of synaptic exocytosis [40,41]. Several misfolded proteins such as huntingtin [42–46], PrP [47], α-synuclein [48] and ataxin 1&3 [49,50] are known substrates for the Hsp40/Hsc70 system. It is tempting to attribute a key role to the association of Hsp40 with the CSP40/Hsc70 chaperone complex to include a transient chaperone activity from dedicated to indiscriminant.

In conclusion, our combined findings suggest a model in which heat shock alters the composition of the CSP40 chaperone complex to include Hsp40. Understanding the composition of the CSP40 chaperone complex, either basal or following a conditioning stress, is crucial to understanding the physiological role(s) of CSP40. Data presented here show that in vitro, Hsp40 increased the CSP40-induced increase in steady state GTP hydrolysis of Gαs. Furthermore, in CAD cells, induction of the heat shock response decreased CSP40 dimerization and increased isoform-specific phosphorylation of synapsin. In view of the crucial importance of stress-induced chaperones in protection against cell death, our data attribute a key role to the association of Hsp40 with the CSP40 chaperone complex in neuroprotection.

Materials and Methods

Reagents and Chemicals

Anti-CSP40 polyclonal was prepared as described previously [13]. Anti-Hsp40 mouse monoclonal, anti-Hsp40 rabbit polyclonal, anti-Hsp90 mouse monoclonal, anti-Hsp25 rabbit polyclonal, anti-weather phosphosynapsin and anti-synapsin rabbit polyclonal antibodies were from Cell Signaling Technology. Anti-c-myc mouse monoclonal was from Clonetech. Anti-actin mouse monoclonal, anti-Hsp70/Hsc70 mouse monoclonal, quercetin and isoproteonol were from Sigma. Geldanamycin was from Calbiochem. 17AA was from Invitrogen. Novobiocin was from EMO Biosciences Inc.

CAD mouse neuroblastoma cells were seeded into 6 well plates and grown in DMEM/F12 medium supplemented with 10% fetal bovine serum and 1% Penicillin/streptomycin. For differentiation cells were grown in Opti-MEM for 60 hrs. LAV1 human neuroblastoma cells were seeded into 6 well plates and grown in RPMI medium supplemented with 10% fetal bovine serum and 1% Penicillin/streptomycin. PC12 cells were obtained from ATCC. PC12 cells were grown in Dulbecco’s modified Eagle’s Medium supplemented with 10% heat-inactivated horse serum and 5% fetal calf serum. For differentiation PC12 cells were treated with 50 ng/ml mouse NGF (R&D systems) for 9 days. Human embryonic kidney tsa-201 (HEK) cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were lysed in 40 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 0.1% SDS, 1% TX100, 0.5 mM PMSF and protease inhibitor (Sigma) end-over-end at 4°C for 1 hr. Lysates were centrifuged at 150,000 x g for 5 min at 4°C and the supernatant was collected. Protein concentration was determined using a Bradford reagent (BioRad).

Transient transfection of CAD cells

CAD cells were washed in PBS and transiently transfected with 0.5 ug myc-tagged rat CSP40 DNA using Lipofectamine-2000 (Invitrogen) in Opti-MEM, and maintained in culture for 24 hrs prior to heat shock or drug treatment.

Cell lines and lystate preparation

Whole rat brains were homogenized in 20 mM Tris-HCl buffer (pH 7.4), 2 mM MgSO4, 1 mM PMSF and EDTA-free inhibitor cocktail as previously described [51]. The homogenate was centrifuged at 100,000 x g for 1 hr at 4°C. The resultant soluble fraction was removed and designated the soluble cytosolic fraction (S). The remaining pellet was solubilized in homogenizing buffer containing 1% (w/v) n-dodecyl-β-D-maltoside (Calbiochem) for 60 min at 4°C. Following centrifugation at 100,000 x g for 1 hr at 4°C, the resulting supernatant constituted the detergent-solubilized membrane particulate fraction (P). All procedures were carried out in strict accordance with a protocol approved by the University of Calgary Animal Care Committee.

Preparation of fusion proteins

Glutathione-S-transferase (GST) fusion proteins GST-Hsp40, GST-CSP40, GST-CSP40 HPD and GST-Hsc70 were prepared by sub-cloning PCR products into the bacterial expression plasmids pGEX-KG or pGEX-IT, as previously described [13,51]. Hsp40HPD-AAA, Hsp401-80, Hsp401-80 HPD-AAA mutants were prepared by subcloning restriction or PCR fragments into the bacterial expression plasmid pGEX-KG. Following sequence verification, DNA was transformed into AB1899 or DH5α strain of Escherichia coli. Expression of GST fusion proteins was induced with 100 μM isopropyl-β-D-thiogalactoside (IPTG) for 5 hours at 37°C. Bacteria were suspended in PBS, 0.05% (v/v) Tween 20, 2 mM EDTA, 0.5 mM PMSF and 0.1% (v/v) β-mercaptoethanol and lysed by two passages through a French press (Spectronics Instruments Inc.) GST fusion proteins were recovered by binding to glutathione–sepharose beads (GE Healthcare Bionsciences). Beads were suspended as a 50% (v/v) slurry in 20 mM MOPS, 45 mM Mg acetate, 150 mM KCl, and 0.2% (v/v) Triton X-100. The concentrations of recombinant GST fusion proteins were estimated by Coomassie blue staining of SDS-polyacrylamide gels using bovine serum albumin (BSA) as a standard. The recombi-
nant portion of the fusion protein was eluted from the beads with 50 mM Tris (pH 7.5), 150 mM NaCl, 2.5 mM CaCl₂, 0.1% β-mercaptoethanol and 3 μg/ml thrombin.

**In vitro ‘Pull-down’ Assays**

For the ‘pull down assays’, cells were lysed end-over-end in 20 mM Tris (pH 7.4), 130 mM NaCl, 2 mM MgSO₄, 2 mM NaVO₄, 1% n-dodecyl-β-D-maltoside, 1 mM PMSF and protease inhibitor (Sigma) for 60 min at 4°C. Lysates were centrifuged at 20,000×g for 30 minutes and the total detergent-solubilized cell lysate (supernatant) was collected. Protein concentration was determined using a Bradford reagent (BioRad). The detergent-solubilized cell lysates were incubated with bead-immobilized GST-tagged proteins in 20 mM MOPS, 4.5 mM Mgacetate, 150 mM KCl, 0.5% Tx100, and 2 mM ATP or 2 mM GDP in a final volume of 400 μl, for 1 hour at 37°C. Beads were washed...
twice with 200 μl of ice cold 20 mM MOPS, 4.5 mM MgAcetate, 150 mM KCl, 0.2% Tx100. Bound proteins were eluted in Laemmli sample buffer, fractionated by SDS-PAGE and analyzed by Western blotting.

**Immunoblotting**

Proteins were transferred from polyacrylamide gels to nitrocellulose (0.45 μm) in 20 mM Tris, 150 mM glycine, and 12% methanol. Membranes were blocked with 4% milk solution (prepared in PBS with 0.1% Tween 20) and incubated with primary antibody for 2 hours at room temperature or overnight at 4°C. The membranes were washed in blocking solution and incubated with horseradish peroxidase-coupled secondary antibody. The signal was developed using West Pico Pierce reagent (Pierce Biotechnology, Inc.) and exposed to Kodak film.

**Sequence analysis** CSPα from *Rattus norvegicus* (accession number: NP_077075.1) and Hsp40 from *Rattus norvegicus* (accession number: EDI.92267.1) were evaluated for homology between J domains. Alignments of sequences were obtained using CLUSTAL-W with default settings in place and EMBOSS pairwise global alignment using an implementation of the Needleman-Wunsch algorithm [52]. Domains were identified with InterProScan and secondary structure prediction was carried out using PredictProtein [53].

**Immunofluorescence**

CAD cells were grown on coverslips coated with glycerol in DMEM/F12 or Opti-MEM for differentiation. Cells were washed
in PBS, fixed in 2% paraformaldehyde for 10 min, permeabilized in ice cold methanol for 10 min and rinsed with PBS. Cells were blocked in a 3% BSA, 0.05% Tween 20 solution in PBS for 30 min. Incubations of cells with primary antibodies were carried out sequentially overnight at 4°C and 1 hr at room temperature. Following incubation with primary antibody, cells were washed 3× with PBS and incubated with either goat anti-mouse conjugated to Alexa 488 or sheep anti-rabbit conjugated to Cy3 secondary antibody in the blocking solution for 1 hr at room temperature. Following secondary antibody incubation, cells were washed 3× with PBS, mounted onto glass slides with DABCO (Sigma) and photographed with a Leica confocal microscope. The fluorophores Alexa 488 and Cy3 were excited at 470 nm and 555 nm respectively, and images were collected at 525 nm and 610 nm.

GTPase assay

Steady-state GTPase reactions were performed at 25°C in the presence of Gαs (0.3 μM), Gβγ (0.6 μM), and the absence or presence of 0.3 μM HsP40, CSPα, Hs70 and SGT [54]. Proteins were mixed with 10 μM [γ-32P]GTP (1μCi) in a final volume of 50 μl of 20 mM Tris-HCl (pH 8.0) buffer containing 130 mM NaCl and 10 mM MgSO4 and the reaction was started by the addition of Gαs. Aliquots (20 μl) were withdrawn after 30 min and transferred to 100 μl of 7% (v/v) perchloric acid. Nucleotides were precipitated with 700 μl of 10% (w/v) charcoal suspension in phosphate-buffered saline and free [32P] was measured with liquid scintillation counting. Results were fitted with linear regression.

Supporting Information

**Figure S1** Comparison of the amino acid sequences and predicted secondary structures of Rat CSPα and Rat Hsp40. (A) The locations of J domain and DnaJ C-terminal are highlighted in magenta and cyan respectively; the cysteine repeat region of CSPα_Rat is in red background. Alignments of sequences were obtained using ClustalW with default settings in place. (B) InterProScan and PredictProtein were used to identify the domains and secondary structures. Scale bar marks the length measured by amino acids.

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**Figure S2** Western analysis showing the expression of CSPα, Hsp40 and Hsp70 in PC12 and CAD cells treated with the indicated agents. (A) undifferentiated PC12 cells. PC12 cell cultures were treated with 200 μM quercetin, 500 nM 17-AAG, 2 μM geldanamycin, 50 μM isoproterenol, 50 ng/ml NGF, 50 mM ethanol, 0.1% v/v DMSO, 1 mM LCl or 200 μM novobiocin for 4 days. (B) Time course of geldanamycin (2 μM) response in PC12 cells. (C) Time course of geldanamycin (2 μM) response in CAD cells. Hsp70, Hsp40, CSPα, Hsp25 and actin were detected by Western analysis. (D) CAD cells were treated with geldanamycin (1 μM) for 24 hrs and probed for CSPα expression. Quantification of CSPα is shown. Data were derived from a total of 7 separate experiments.

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Author Contributions

Conceived and designed the experiments: NOA JEAB. Performed the experiments: SJJ BB JP TN. Analyzed the data: SJJ BB APB NOA JEAB. Contributed reagents/materials/analysis tools: KEB XZ APB. Wrote the paper: JEAB.

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