DJA1 and DJA2 Carboxyl-Terminal Fragments and their Role in Peptide Binding and Luciferase Refolding

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Abstract

DJA1 and DJA2 are Hsp40-family co-chaperone proteins that regulate the activity of the chaperone Hsc70/Hsp70. The N-terminal J domains of DJA1 and DJA2 promote ATP hydrolysis and polypeptide binding by Hsc70. Both co-chaperones are also thought to bind unfolded polypeptides in their central to C-terminal regions. Yet, the two are functionally distinct, possibly due to divergence in the connections between their functional domains. Here, we constructed C-terminal fragments of DJA1 (A1-C, residues 254-397) and DJA2 (A2-C, residues 254-412), expected to contain the homodimerization site but not the main polypeptide-binding region. The fragments were expressed in *E. coli* under optimized conditions, and purified. Co-precipitation experiments with the pure fragments suggested that A1-C retained partial binding to model polypeptides, while A2-C showed less binding. The fragments were then transfected into HeLa cells, in which overexpression of full-length DJA1 and DJA2 had previously been found to increase the folding of a co-transfected model protein. Both A1-C and A2-C increased folding moderately, less than the full-length co-chaperones, but notably the fragments did not inhibit folding. These results suggest that these C-terminal domains may provide a second site for polypeptide binding, contributing to the substrate specificity or overall activity of the co-chaperones.

Glossary

Hsp70: Heat shock protein 70kDA; a chaperone
DJA: DnaJ domain-containing co-chaperone of Hsp70
A1/2-C: Protein fragment of corresponding DJA protein containing only the C-terminal domain
NEF: Nucleotide Exchange Factor

Keywords

Chaperone, DnaJ co-chaperones, Hsp40, Hsp70, DJA1, DJA2, DNAJA1, DNAJA2

Introduction

Molecular chaperones are considered key players in a variety of events related to protein metabolism. Chaperones display increased expression under conditions of cellular stress and are believed to mediate folding and prevent aggregation of proteins. They also play important roles in protein translation, translocation and degradation.

One of the most important chaperones, Heat Shock Protein 70 (Hsp70), is a ubiquitous chaperone that appears to be particularly important in genital development (Dix, *et al*. 1996). Hsp70 chaperones bind to hydrophobic regions of unfolded polypeptides and prevent the formation of non-productive aggregates while promoting polypeptide folding. Although the exact mechanism by which it promotes folding is still under contention, it is believed that Hsp70 acts as a molecular wrench to pull protein segments apart during folding to allow the polypeptide to re-nature the hydrophobic region that is affected (Goloubinoff, *et al*. 2007). A cycle of ATP binding, hydrolysis and nucleotide exchange controls this refolding activity (Qiu, *et al*. 2006). When bound to ATP, Hsp70 has a low affinity for unfolded polypeptides, but hydrolysis of ATP to ADP leaves the Hsp70:ADP complex in a state competent for polypeptide binding. Previous work suggests that nucleotide exchange factors (NEFs) are responsible for triggering Hsp70 dissociation from ADP (Cyr, *et al*. 2008), while the J-domain of co-chaperones from the DnaJ/Hsp40 family appears to stimulate hydrolysis of ATP (Tzankov, *et al*. 2008). The first biochemical and genetic evidence to support this mechanism of ATPase regulation by DnaJ proteins was identified in *Escherichia coli*, however, much of our understanding of J-protein function comes from work performed on Ydj1, the DJA1 equivalent in *Saccharomyces cerevisiae*.

Between lower and higher eukaryotes, there is significant sequence conservation in both the J-domain and the C-terminal domain (Figure 1) (Cheetham, *et al*. 1998). Crystal structures of Ydj1 segments suggest that the C-terminal domain of DnaJ proteins is involved in dimerization (Wu, *et al*. 2005) and may also play a role in binding short peptide sequences (Li, *et al*. 2003). As well, research on Ydj1's functional domains has proven valuable in elucidating the diverse spectrum of roles that the human DnaJ homologs play within the cell (Ramos, *et al*. 2008).

One structural modification that is shared between DNAJA1, DNAJA2 and Ydj1 is a farnesylation motif. Located at the C-terminus, the farnesyl group is covalently attached to a cysteine residue by a thioester bond and mimicks the hydrophobic qualities of a lipid due to its highly aliphatic, conjugated structure (Wright and Philips, 2006). Although the significance of this modification in Hsp40 homologs is still not clear, farnesylation is often used to target proteins to membranes. A recent paper by Flom, *et al*. suggests that disruption of farnesyltransferases disrupts Ydj1 interaction with Hsp90 chaperones, however, this data has yet to be confirmed in mammalian cells (Flom, *et al*. 2008).

More than 20 DnaJ proteins have been reported in humans and these can be divided into three groups based on the presence or absence of certain functional domains. Type I Hsp40 proteins have the same structural design as DnaJ; a J-domain located at the amino-terminus, followed by a linker region rich in glycine and phenylalanine, a zinc-finger region and a carboxyl-terminal homodimerization domain. Type II proteins lack the zinc-finger region, while Type III proteins consist only of a J-domain. In bacteria, DnaJ is the only J-domain co-chaperone of DnaK, the Hsp40 homolog. In humans, however, there are three cytosolic Type I co-chaperones, DJA1, DJA2 and DJA4. DJA4 and the Type II co-chaperones appear to have either specialized functions or increased levels of synthesis under stress conditions, whereas DJA1 and DJA2 are expressed constitutively in all cells (Terada, *et al*. 2000). Accordingly, DJA1 and DJA2 appear to be the main regulators of Hsp70.

Recent work from this laboratory suggests a more complex Hsp70/Hsp40 relationship than the DnaJ/DnaK presen-

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DNAJA1          DQKDHAVFTRRGEDLFMCMDIQLVEALCGFQKPISTLDNRTIVITSHPGQIVKHGDIKCV 303
DNAJA2          -EKEHEVFQRDGNDLHMTYKIGLVEALCGFQFTFKHLDGRQIVVKYPPGKVIEPGCVRVV 312
YDJ1            ------SFKRDGDDLVYEAEIDLLTAIAGGEFALEHVSGDWLKVGIVPGEVIAPGMRKVI 311
                    *  *  *:**       .*  *: *:.*  :  .:.  :..    : :     **:::   *   :  :

DNAJA1          LNEGMPIYRRPYEKGRLIIEFKVNFPENGFLSPDKLSLLEKLLPERKE-VEETD-EMDQV 361
DNAJA2          RGEGMPQYRNPFEKGDLYIKFDVQFPENNWINPDKLSELEDLLPSRPE-VPNIIGETEEV 371
YDJ1            EGKGMPIPKYGG-YGNLIIKFTIKFPENHFTSEENLKKLEEILPPRIVPAIPKKATVDEC 370
                 .:***   :        *  *  *:*  ::****  :  .  ::*.  **.:** *    .            ::

DNAJA1          ELVDFDPNQ-----ERRRHYNGEAYEDDEHHPRGGVQCQTS 397
DNAJA2          ELQEFDSTRGSGGGQRREAYNDSSDEESSSHHGPGVQCAHQ 412
YDJ1            VLADFDPA--------------------------------- 378
                 *  :**.
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Figure 1: Protein sequence alignment of C-terminal dimerization segments of human DJA1, DJA2 and yeast Ydj1, the homolog of the human DJA1. DJA1 and DJA2 share 48% homology and are 33% and 38%, respectively, homologous with Ydj1 in their C-terminal dimerization sequences.

ted in the bacterial model. Clear variations in biochemical properties have been shown amongst DJAs. DJA1 appears to activate the ATPase domain of Hsc70 the strongest and has the highest binding affinity to preproteins. Furthermore, DJA1 function *in vivo* has been shown to be the most effective at promoting luciferase renaturation and the mitochondrial import of phosphate carrier protein (PiC). DJA2 promotes luciferase refolding with the slightly less efficiency than DJA1. A DJA2 mutant lacking the J-domain (ΔJ) inhibits binding of Hsc70-preprotein most severely (Bhangoo, *et al.* 2007). Previous results from our laboratory suggest that this partial specialization allows assistance of a wider range of substrates and, hence, provides the cell with a greater advantage over a cell possessing a single Hsp40 co-chaperone.

It has since been determined that DJA1 and DJA2 are functionally distinct with reference to their ATPase activating domains and peptide-binding domains, although contributors to these differences have yet to be established (Tzankov, *et al.* 2008). The differences could be due to divergence in the connections between their functional domains. The C-terminal homodimerization domains of the DJAs may be important for functional connections and, furthermore, may have additional interactions with substrate polypeptides or even other chaperones. To investigate these ideas, C-terminal fragments of DJA1 and DJA2 carboxyl-terminus mutants were studied for their ability to bind to different polypeptides and to affect luciferase folding in live cells.

Methods
Plasmids
Sequences encoding DJA1 C-term (amino acids 254-397) and DJA2 C-term (amino acids 254-412) were amplified by PCR using pcDNA3.1 DJA1-mycHis and pcDNA3.1 DJA2-mycHis as templates. The following specific primers were used (Integrated DNA Technologies, Inc.); DJA1 244C Bam up (ccggccggatccgatgGATCAGAAGGACCATGCTG), DJA1 MHC dn (cggccagcggccgcagaggtctgacactgaacacc), DJA2 254 Bam up (ccggccggatccgatgGAGAAAGAACATGAGGTATTTC), and DJA2 MHC dn (cggccagcggccgcctgatgggcacactgcactcc). PCR products were digested with restriction enzymes, BamHI and NotI (New England Biolabs) and inserted into cut pcDNA3.1 mycHis C with T4 ligase. Plasmids were transformed into DH5α cells and DNA was extracted using a QIAprep Spin Miniprep Kit (250). To identify positive clones, the DNA was cut with BamHI and NotI, and resolved on a 1% polyacralymide gel. DNA purification using QIAGEN Plasmid Midi Kits gave final DNA concentrations of 1.03 μg/μl and 1.09 μg/μl for DNA1 and DJA2 mutants, respectively. The same procedure was used for cloning C-terminal fragments into pPROEXHTA (Invitrogen), with the following exceptions: primers used were

DJA1 PRO new dn (ggccggctcgagtcaagaggtctgacactgaac) and DJA2 Sal dn (ggccgggtcgacttactgatgggcacactgc), and PCR products and vectors were digested with BamHI, XhoI, and BamHI, SalI for DJA1 and DJA2 mutants respectively. Final DNA concentrations were 0.14 μg/μl and 0.13μg/μl for DJA1 and DJA2 mutants, respectively.

Full length mycHis-tagged DJA in pcDNA 3.1 (Invitrogen), DJA ΔJ mycHis-tagged mutants in pcDNA 3.1, along with PiC in pGEM-SP6 were as previously reported (Bhangoo, *et al.* 2007; Tzankov, *et al.* 2008).

Bacterial Expression
Cultures of DJA1 and DJA C-term in pPROEXHTA plasmids were incubated at 37˚C until the measured OD of 0.8-1.0 was reached in BL21DE3 cells (Promega). IPTG was added to a final concentration of 1mM to induce expression and cultures were grown at 37˚C for 30, 60 and 90 minutes and 30˚C for 1, 2 and 3 hours. Once the induction time was completed, cells were harvested at 8000xg for 10 minutes, followed by resuspension in DJA equilibration buffer (750mM, 60mM imidazole, 20mM KH_xPO_4 pH 7.5) (Bhangoo, *et al.* 2007) with Complete Protease Inhibitors (Roche Diagnostics).

Small Scale Condition Experimentation for Protein Purification
Aliquots were lysed by addition of lysozyme to a final concentration of 0.5mg/mL, followed by 30 minutes of incubation on ice. Tween-20 and DNase1 were added to final concentrations of 0.1% and 33μg/mL, respectively, then allowed to incubate on ice for 30 minutes. Samples were pelleted at 20,000 x *g* for 1 hour and rocked with 50μL nickel-Sepharose columns (GE Healthcare) at 4˚C for 30 minutes. After washing with DJA equilibration buffer, two fractions were collected with an appropriate elution buffer (1M imidazole, 0.5M NaCl, 20mM KH_xPO_4 pH 7.5) (Bhangoo, *et al.* 2007).

Full Scale Purification
Aliquots of suspended bacterial pellet were lysed by cavitation in a french press then pelleted at 20,000 rpm for 1 hour. The supernatant was run through a 5mL high-performance nickel-Sepharose column and proteins were eluted using an elution buffer. Fractions containing protein peaks were loaded onto a Superdex 200 Hi-Load 16/60 column (GE Healthcare) and eluted with Hi-Salt buffer (500 mM NaCl, 20 mM HEPES-KOH, pH 7.5, and 5 mM $MgOAc_2$). Yield of peak fractions was determined by BCA assay.

Coprecipitation Experiments
The same assay as previously outlined for quantifying binding of pre-proteins was used (Bhangoo, *et al.* 2007, from Young, *et al.* 2003; Fan, *et al.* 2006). Purified DJA1, DJA2 and their respec-

tive C-terminal mutants at final concentrations of 5μM were bound to nickel-Sepharose in buffer H (500mM NaCl, 20mM HEPES-KOH at pH 7.5, 5mM $MgOAc_2$) for 30 minutes at 4°C. Rabbit reticulocyte lysate expression systems were used to translate the phosphate carrier (PiC) and oxoglutarate carrier (OGC) (Palmisano, A. *et al.* 1998) with SP6 polymerase, diluted 1:20 into buffer GTI (20mM imidazole, 0.1% Triton X-100, 500mM NaCl, 20mM HEPES-KOH pH 7.5, 5mM $MgOAc_2$) with 2mg/mL ovalbumin. Protein reactions were terminated with 0.1U/μL apyrase after 2 minutes at room temperature. DJA-protein complexes were recovered on beads for 30 minutes at 4°C then washed with buffer GTI. All washing steps were conducted with Buffer G (100mM KOAc, 20mM HEPES-KOH pH 7.5, 5mM $MgOAc_2$). Complexes were eluted using Laemmli loading buffer and 50mM EDTA, then analyzed on 12% SDS-PAGE and Typhoon Phosphimager (GE Healthcare).

Construct Transfection and Luciferase Assays
HeLa cells were maintained in DMEM containing 4.5 g/l glucose, 36 mg/l pyruvate, 2mM glutamine, and 10% fetal bovine serum (Invitrogen). Cells were grown to a density of 2.5×10^5 cells/ml, and co-transfected using 2 μl of Lipofectamine 2000 PLUS (Invitrogen) in twelve-well plates with either 1.6 μg of

DJA C-term mycHis, DJA ΔJ mycHis, the full length mycHis DJA, or vector alone, and 0.4 μg of HA-tagged luciferase. All of these DNA samples were cloned in pcDNA 3.1. Twenty-four hours after transfection, cells were harvested with 0.4mL 1X PBS, centrifuged at 600 x *g* for five minutes and lysed using 80 μL of 1% Triton-X 100 in 1X PBS. Lysates were left at 4°C for four minutes and cleared by centrifugation at 20,000 x *g* for five minutes. Lysates were assayed for luciferase activity using luciferase reagent (Promega) in a 1:5 dilution and RLU/sec measurements were recorded using a SIRIUS Luminometer V3.2. Equal amounts of lysate were resolved by 12% SDS-PAGE and analyzed with immunoblots using α-HA (Upstate Cell Signaling Solutions) and 9E10 α-c-Myc (Covance) as primary antibodies, and α-mouse-HRP (Stressgen Bioreagents) as secondary antibody. Signals were observed on HyBlot CL autoradiography film using ECL reagent (GE Healthcare) with various exposure times, ranging from three seconds to three minutes. Protein concentration was assayed using BCA reagents (Thermo Scientific) and 10 μL of cell lysate in a 1:500 dilution. Optical Density (OD) measurements at 562 nm were taken using a Beckman Coulter DU 730 UV/Vis Spectrophotometer; these readings were used to calculate the normalized luciferase activity.
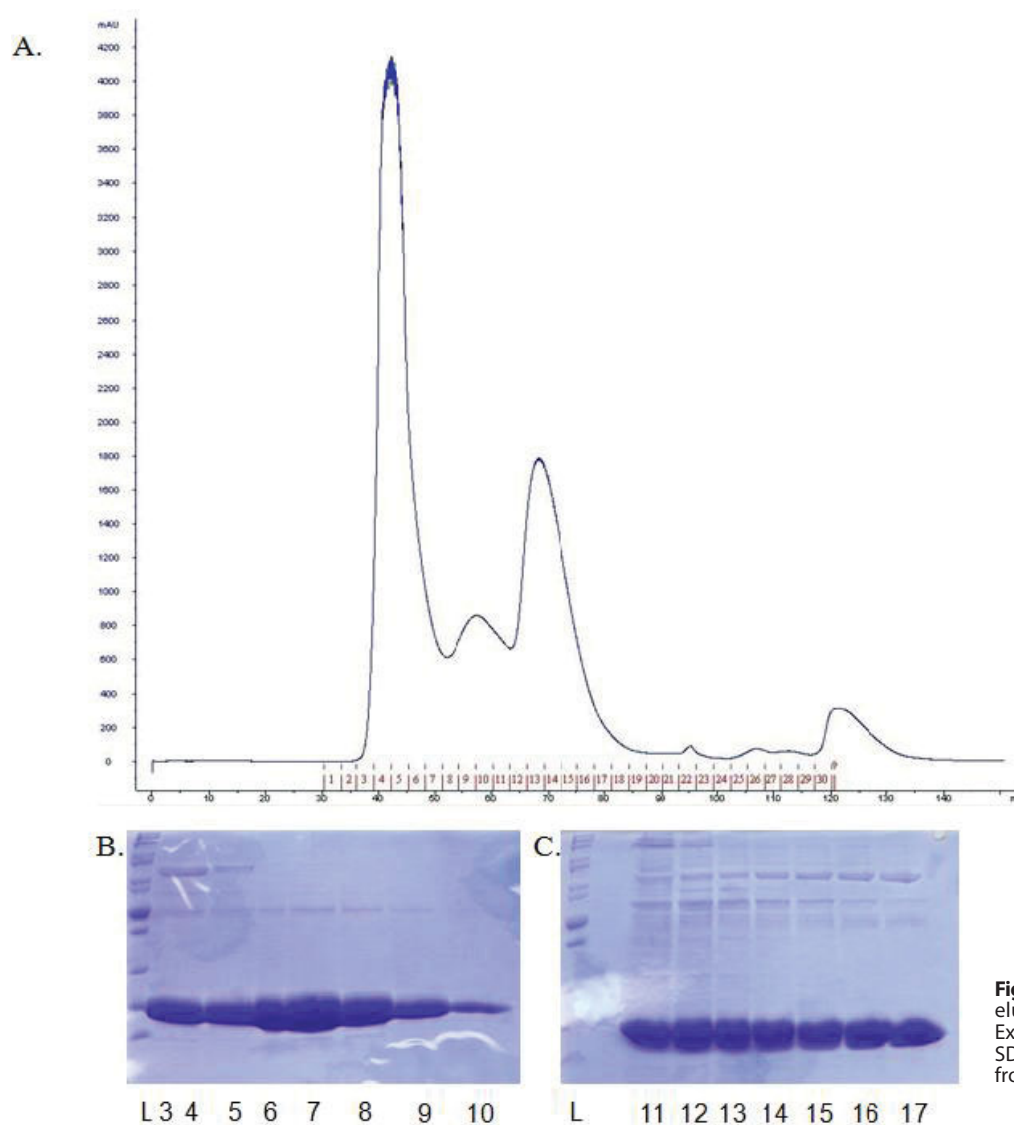
Figure 2: (A) The UV profile of DJA1 C-term elution fractions from Superdex Size-Exclusion chromatography column and SDS-PAGE analysis of fraction content from fractions (B) 3-10 and (C) 11-17.

Results

Isolation of DJA1 and DJA2 C-terminal Mutants

The predicted C-terminal homodimerization domains of DJA1 and DJA2 (A1-C and A2-C, respectively) are well conserved, although not identical (Figure 1). To study their possible functions and properties, protein fragments of the C-termini of DJA1 and DJA2 fused to N-terminal His-tags were first expressed in *E. coli*, then purified.
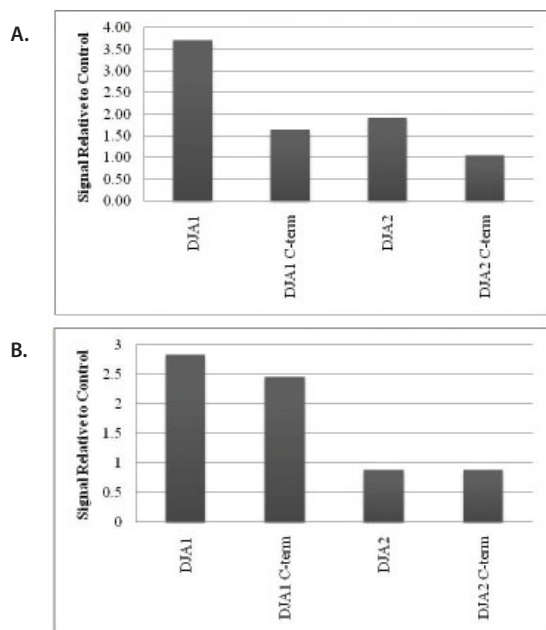
To determine the optimal conditions for bacterial expression, induction at 37°C and 30°C was tested at several time points. At each temperature/time point, the bacteria expressing A1-C and A2-C were lysed and single step purifications on nickel-Sepharose were carried out. SDS-PAGE gel analysis of pellet, flow-through, wash and two eluates was conducted for both C-terminal proteins. Expression profiles were selected for magnitude of the expected product band and significance of contaminating agents in eluates. In most conditions, the proteins appeared to express well, although there were differences in amount and number of contaminants after the trial purification. For A1-C and A2-C, it was determined that the optimal conditions for large scale purification were 30°C for 2 hours and 37°C for 1 hour, respectively.

The A1-C and A2-C proteins were next purified on a larger scale to produce amounts sufficient for *in vitro* experiments. After expressing the proteins under the conditions described above, a first purification step using a nickel-Sepharose column was performed. As expected, the proteins eluted in a narrow peak and appeared relatively pure. These fractions were pooled and loaded on a Superdex 200 gel filtration column, producing broad elution profiles for both A1-C and A2-C. Some aggregated material eluted in the void volume, but one distinctive peak in the separation range of the column was indicated by the UV absorbance profiles. Interestingly, a smaller earlier peak was observed for both A1-C and A2-C upon gel filtration (Figure 2, and data not shown), suggesting possible formation of tetramers. Because SDS-PAGE gel analysis showed that both peaks contained the correct proteins, fractions combining both peaks were pooled and concentrated. Final concentra-
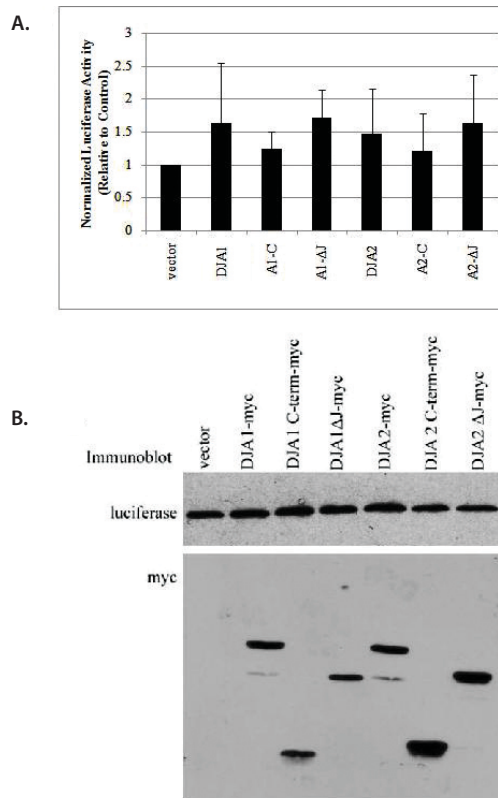
tions of A1-C and A2-C were determined to be 1.22mg/mL and 0.803mg/mL, respectively, when concentrated to 2mL.
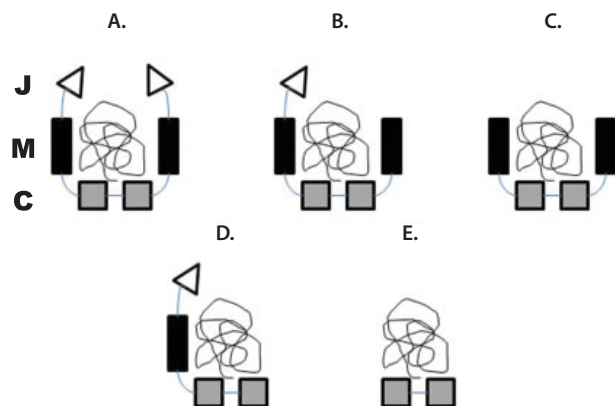
Coprecipitation Experiments

It was possible that the C-terminal fragments of the DJAs contained secondary binding sites for unfolded polypeptides, in addition to the main binding site in the middle domains. To address this, an established assay to test polypeptide binding to the purified full-length His-tagged DJAs was used. The DJAs were known to bind certain unfolded mitochondrial precursor proteins, which depend on the chaperone system for their import, including the phosphate carrier (PiC) and oxoglutarate carrier (OGC). A1-C and A2-C were bound to nickel-sepharose beads in small columns then incubated with RRL translation reactions expressing radiolabelled PiC and OGC. The amounts of radiolabelled polypeptide co-precipitating with the DJA proteins were analyzed. Similar trends were observed for co-precipitation experiments with both preproteins. Positive control experiments using full-length DJA1 and DJA2 were as expected from previous results (Tzankov, *et al.* 2008; Bhangoo, *et al.* 2007). DJA1 displays the strongest binding affinity for PiC at nearly four-fold binding compared to the negative control, whereas full-length DJA2 displays about half the binding efficiency (Figure 3). The C-terminal mutants have decreased PiC binding efficiency relative to their full-length proteins, at levels similar to the negative control. Interestingly, co-precipitation with OGC revealed a different trend to PiC. At just under three-fold binding efficiency relative to negative control,

Figure 4: DJA Mutant function in vivo. HeLa cells were transfected with plasmids expressing luciferase, and either empty vector or the indicated myc-tagged DJA proteins. Twenty-four hours after transfection, cells were harvested. (A) Equivalent amounts of lysate were analyzed for luciferase folding activities. To compare folding activities, the average activities of cells transfected with luciferase and empty vector were set to one. (B) Equivalent amounts of lysate were analyzed by immunoblots to detect myc-tagged DJA proteins, ΔJ fragments, C-terminal fragments and HA-tagged luciferase.

Figure 3: Coprecipitation of (A) PiC and (B) OGC with DJA1, DJA2 and their C-terminal mutants relative to a control containing no purified protein. All binding reactions used final protein concentrations of 5µM.

Figure 5: Model of DJA and fragment structure in vivo and in vitro. (A) Full length DJA1 and DJA2 are present as homodimers with three distinct domains; the J-domain, the middle domain and the C-terminal domain. When transfected in cells, it forms heterodimers with (B) the ΔJ fragments and (D) the C-terminal fragments. Our paper and previous research suggest that when purified, the homodimers formed by (C) the ΔJ DJAs and (E) the C-terminal fragments may both have the ability to bind substrate.

DJA1 exhibits lesser affinity for OGC relative to PiC, but both DJA1 and the A1-C C-terminal fragment appear to be significantly better than DJA2 and A2-C at binding OGC.

DJA Mutant Function in vivo
Previous experiments have demonstrated the ability of DJA co-chaperones to promote polypeptide folding in the native cellular environment (Bhangoo, *et al*. 2007). In this study, we examined the effects of overexpressing DJA1 and DJA2 as well as their C-terminal fragments on the folding of firefly luciferase in HeLa cells. When the wild type DJAs were overexpressed they both appeared to display an increased activity relative to the control (Figure 4). Wild type DJA1 displayed an average relative normalized luciferase activity of 1.63, somewhat less than has been reported by a previous publication from this laboratory (Bhangoo, *et al*. 2007). Also, DJA1 was shown to be more effective at promoting luciferase activity than DJA2 (Bhangoo, *et al*. 2007; Tzankov, *et al*. 2008) and DJA2 produced a 1.47 fold increase over the control. Interestingly, overexpression of the DJA C-term mutants resulted in similar increases in activity. The DJA1 C-term mutant produced a relative luciferase activity of 1.25, while the DJA2 C-term mutant produced a result of 1.21 relative to control. As a further comparison, mutants of DJA1 and DJA2 lacking the N-terminal J domains, but having complete central domains, were also tested in the HeLa cells. These fragments, A1-ΔJ and A2-ΔJ, also promoted luciferase activity at 1.71 and 1.63 fold over the control. Overall, it appears that the C-term DJA mutant co-chaperones are capable of promoting polypeptide folding *in vivo*.

Discussion
In this paper, we have explored the roles of DJA1 and DJA2 both *in vitro* and *in vivo* by comparing them to peptide fragments containing only carboxy-terminal segments. Although it may seem that stimulation of Hsp70 with a DnaJ protein lacking its J-domain would be impossible, our research suggests that C-terminal DJA1 and DJA2 still retain some of the stimulation potential of their full-length proteins. We have also outlined an effective purification method for the C-terminal DJA fragments based on methods outlined by Bhangoo, *et al*.
The DJA1 and DJA2 C-terminal mutants have been shown to display decreased binding affinity for peptide subs-

trates when compared to the full-length proteins. This is an interesting result because previous studies on Ydj1, the yeast homolog of DJA1, suggest that the cysteine rich region and the G/F regions may also contribute to some binding specificity (Yan, *et al*. 1999). This implies that the C-terminal portions must have at least some partial capacity to bind substrate and are, therefore, likely to determine peptide specificity. Although further research needs to be done before drawing any definite conclusions, the differences between DJA1 and DJA2 binding to PiC and OGC support the theory that DnaJ homologues may carry peptide specificities. Further studies should aim to determine what specific sequence within the C-terminus is responsible for specificity, and whether this specificity can be controlled by selectively mutating residues. Being able to control Hsp70 binding partners in cells could be an invaluable tool for therapeutics targeting neurodegenerative diseases.
The transfected DJA C-terminal mutants appear to have reduced refolding of firefly luciferase when compared to the full-length DJA proteins in HeLa cells, but they still increase luciferase activity. They do not act as dominant negatives by inhibiting luciferase folding, as might be expected. This could be explained if heterodimers of the DJAs form upon overexpression of the C-terminal fragments, and these proteins having a single J domain and central region are sufficient for co-chaperone activity (Figure 5). This is supported by the results presented here and by Tzankov, *et al*., suggesting that a heterodimer of a DJA and its fragment lacking a J-domain are sufficient for stimulating activity of Hsp70 in luciferase refolding. We interpret these results as evidence that DJA1 and DJA2 do not homodimerize out of any functional necessity, since this structural feature has shown to be unnecessary for stimulating Hsp70 activity. Instead, the DJAs form dimers to increase their ability to activate Hsp70 ATPase function. However, as evident from the error present in Figure 4, these conclusions should only be used as guidelines for future research. We will need to elucidate whether heterodimers can form between DJA proteins and other proteins by increasing the reproducibility of the *in vivo* data.
We have also outlined the experimental basics necessary to implement the C-terminal DJA1 and DJA2 proteins as experimental models for co-chaperone research. As researchers continue to deconstruct the still poorly understood physical framework of DnaJ proteins, creating a library of mutant and fragmented proteins will ensure they have the tools necessary to experiment effectively. Although further research is required to clearly elucidate the potential of the C-terminal fragments as co-chaperones, we have presented a clear description of their isolation and methods for investigating their peptide binding and luciferase refolding activity.

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