The Molecular Chaperone Function of the Secretory Vesicle Cysteine String Proteins*

Luke H. Chamberlain‡ and Robert D. Burgoyne§

From The Physiological Laboratory, Liverpool University, P.O. Box 147, Crown Street, Liverpool L69 3BX, United Kingdom

The "J" domains of eukaryotic DnaJ-like proteins specify interaction with various Hsp70s. The conserved tripeptide, HPD, present in all J domains has been shown to be important for the interaction between yeast and bacterial DnaJ/Hsp70 protein pairs. We have characterized mutations in the HPD motif of the synaptic vesicle protein cysteine-string protein (Csp). Mutation of the histidine (H43Q) or aspartic acid (D45A) residues of this motif reduced the ability of Csp to stimulate the ATPase activity of mammalian Hsc70. The H43Q and D45A mutant proteins were not able to stimulate the ATPase activity of Hsc70 to any significant extent. The mutant proteins were characterized by competition assays, tryptic digestion analysis, and direct binding analysis from which it was seen that these proteins were defective in binding to Hsc70. Thus, the HPD motif of Csp is required for binding to Hsc70. We also analyzed the interaction between Csp and a model substrate protein, denatured firefly luciferase. Both Csp1 and the C-terminally truncated isoform Csp2 were able to prevent aggregation of heat-denatured luciferase, and they also cooperated with Hsc70 to prevent aggregation. In addition, complexes of Csp1 or Csp2 with Hsc70 and luciferase were isolated, confirming that these proteins interact and that Csp1 can bind directly to denatured proteins. Csp1 and Csp2 isoforms must differ in some aspect other than interaction with Hsc70 and substrate protein. These results show that both Csp1 and Csp2 can bind a partially unfolded protein and act as chaperones. This suggests that Csp1 and Csp2 can bind a partially unfolded protein and act as chaperones. To anchor the protein to synaptic vesicle and other regulated secretory vesicle membranes. The exact role of Csp1 is unknown.

We previously cloned two Csp variant proteins from bovine adrenal chromaffin cells (5), and the same two isoforms have been identified from human cDNAs (6). Csp1 is homologous to other identified mammalian Csp1 (3, 4); Csp2 is identical to Csp1, except for a 31-amino acid C-terminal deletion. Csp1 is present in the large dense-core granules of chromaffin cells (9), pancreatic zymogen granules (4), and secretory granules of the neurohypophysis (10), implicating Csp1 in dense-core granule exocytosis, in addition to neurotransmitter release. Csp2 could not be detected in chromaffin cells by Western blotting suggesting that this protein is only expressed at low levels. Csp2 was, however, detected in a spleen homogenate (5). In addition, Csp2 has recently been shown to be enriched in the olfactory bulb and cerebellum of adult rats (16).

The Csp J domain is homologous to the archetypal J domain of the bacterial protein DnaJ. DnaJ interacts with the chaperone protein DnaK (the Escherichia coli equivalent of Hsp70), and these proteins, together with the nucleotide exchange factor, GrpE function in a number of cellular processes, such as protein folding reactions (both under stress and normal conditions) and replication of bacteriophage λ (17). DnaK is an ATPase, and this activity is stimulated by DnaJ, whereas GrpE acts to exchange ADP bound by DnaK for ATP (18).

A number of eukaryotic proteins have J domains, and these proteins are collectively referred to as DnaJ-like proteins. These DnaJ-like proteins can interact with the chaperone protein Hsp70 and its homologues, which are the equivalent of the bacterial DnaK protein, although their interactions have been much less extensively characterized than those of the bacterial protein. In a manner analogous to the prokaryotic system, DnaJ-like proteins can stimulate the intrinsic ATPase activity of Hsp70s. The conserved tripeptide, HPD, present in all J domains, has been shown to be important in the interaction between DnaJ and DnaK (19) and between the yeast DnaJ/ Hsp70 protein pairs Ydj1/Ssa1 (20) and Sec63/Kar2p (21). Since there has been belief to specificity of interaction between specific DnaJ-like proteins and Hsp70 proteins, it would be important to determine the extent to which the HPD motif is required in all such interactions such as those involving mammalian homologues.

In addition to interacting with each other, DnaJ and DnaK/ Hsp70 also interact with substrate proteins which may be in a native or unfolded conformation. The actions of DnaJ/DnaK in mediating correct folding of nascent polypeptides and refolding of denatured proteins is well documented (for review see Ref. 17). However, these proteins also interact with native proteins. For example, Hsc70 (the constitutively expressed mammalian Hsp70) and auxilin (a DnaJ-like protein) interact with clathrin and catalyze the disassembly of this protein from coated vesi-
cles (an essential reaction in synaptic vesicle recycling) (22). In addition, the bacterial proteins Dnaj and DnaK interact with the αP replication protein and in an ATP-dependent reaction release this protein from the preprimosomal complex assembled at the ortha site. This is an essential reaction in the replication of bacteriophage λ DNA (23–25). The interaction of CspS with native or denatured protein substrates has not been examined.

We and others (13, 14) have previously shown that Csp interacts with Hsc70 and can stimulate the ATPase activity of the chaperone protein severalfold. Both Csp1 and Csp2 can stimulate the ATPase activity of Hsc70 to a similar extent (14). There are important questions that arise concerning the interaction between CspS and Hsc70 and the ability of CspS to act as molecular chaperones by interacting with denatured protein substrates. First, is the conserved HPD motif in CspS required for Csp activation of Hsc70 ATPase activity? Second, is the HPD motif required for binding of Csp to Hsc70? Third, can CspS interact as chaperones with denatured protein substrates? Fourth, do the Csp1 and Csp2 isoforms differ in their interaction with denatured protein substrates? Analysis of these questions is necessary to establish the extent to which the secretory vesicle CspS function in a chaperone cycle with Hsc70 and their similarity or otherwise to the well-characterized bacterial Dnaj/DnaK proteins. In addition, understanding of these protein-protein interactions and assessment of the ability of CspS themselves to act as chaperones may help to understand the function of CspS in exocytosis.

EXPERIMENTAL PROCEDURES

Materials—Ni2+-NTA agarose, pQE30 expression vector, and E. coli M15 cells were obtained from Qiagen (Surrey, UK). Supercompetent XL1 blue cells and QuickChange site-directed mutagenesis kit were purchased from Stratagene (Cambridge, UK). Restriction enzymes were purchased from Promega (Madison, WI). Enhanced chemiluminescence antibody-binding detection kit was obtained from Amersham Corp. (Slough, UK). Firefly luciferase IgG was purchased from Europa Ltd. (UK). Firefly luciferase, Hsc70 antiserum, and all other chemicals were of an analytical grade from Sigma (Poole, UK).

Generation of Plasmid Constructs—The coding sequences of Csp1 and Csp2 were amplified by the polymerase chain reaction from bovine adrenal chromaffin cell cDNA and cloned into the pQE30 expression vector (Qiagen) as described previously (5). The Csp1-pQE30 construct was used as a template for several site-directed mutageneses (QuickChange; Stratagene). The sense and antisense primers used for mutation of amino acid His433 were 5′-CTACCGGAAGCT(G/A)GCCTTGAAATACCA(G/C)-GAAATACCACCCTG(A/C)CAAGAACCCTG and 5′-CTACCGGAAGCT(G/A)GCCTTGAAATACCA(G/C)-GAAATACCACCCTG(A/C)CAAGAACCCTG and 5′-CTACCGGAAGCT(G/A)GCCTTGAAATACCA(G/C)-GAAATACCACCCTG(A/C)CAAGAACCCTG respectively. The sense and antisense primers used for mutation of amino acid Asp508 were 5′-CTACCGGAAGCT(G/A)GCCTTGAAATACCA(G/C)-GAAATACCACCCTG(A/C)CAAGAACCCTG and 5′-CTACCGGAAGCT(G/A)GCCTTGAAATACCA(G/C)-GAAATACCACCCTG(A/C)CAAGAACCCTG respectively. The mutation (sense primers) was included to introduce an M15 E. coli recognition site to allow screening of mutant constructs. This mutation was conservative and, therefore, did not alter the amino acid sequence of Csp1. The sense and antisense primers used for mutation of amino acid His433 were 5′-CTACCGGAAGCT(G/A)GCCTTGAAATACCA(G/C)-GAAATACCACCCTG(A/C)CAAGAACCCTG and 5′-CTACCGGAAGCT(G/A)GCCTTGAAATACCA(G/C)-GAAATACCACCCTG(A/C)CAAGAACCCTG respectively. The sense and antisense primers used for mutation of amino acid His433 were 5′-CTACCGGAAGCT(G/A)GCCTTGAAATACCA(G/C)-GAAATACCACCCTG(A/C)CAAGAACCCTG and 5′-CTACCGGAAGCT(G/A)GCCTTGAAATACCA(G/C)-GAAATACCACCCTG(A/C)CAAGAACCCTG respectively. The sense and antisense primers used for mutation of amino acid Asp508 were 5′-CTACCGGAAGCT(G/A)GCCTTGAAATACCA(G/C)-GAAATACCACCCTG(A/C)CAAGAACCCTG and 5′-CTACCGGAAGCT(G/A)GCCTTGAAATACCA(G/C)-GAAATACCACCCTG(A/C)CAAGAACCCTG respectively. The sense and antisense primers used for mutation of amino acid Asp508 were 5′-CTACCGGAAGCT(G/A)GCCTTGAAATACCA(G/C)-GAAATACCACCCTG(A/C)CAAGAACCCTG and 5′-CTACCGGAAGCT(G/A)GCCTTGAAATACCA(G/C)-GAAATACCACCCTG(A/C)CAAGAACCCTG respectively.

Expression and Purification of Proteins—Isopropyl-1-thio-galactopyranoside (2 mM) was added to a 1-liter culture of E. coli M15 cells containing the cloned csp1 and csp2 sequences (at A900 = 0.7–0.9) and incubated at 37 °C for 5 h with shaking at 230 rpm, inducing expression of the recombinant proteins. The induced cells were washed and resuspended in 20 ml of breaking buffer (100 mM HEPEs, 500 mM KC1, 5 mM MgCl2, 5 mM ATP, 2 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 1 μg/ml pepstatin, pH 7). The cells were stored at −80 °C overnight and thawed the following morning. 1 mg/ml lysozyme was added and the cells left on ice for 30 min. Following ultrasonication, 2 μg/ml DNase was added, and the cells were left on ice for 15 min. Centrifugation at 100,000 × g removed cell debris, and the supernatant was loaded onto a Ni2+-NTA agarose column and washed with 50 mM imidazole buffer (50 mM imidazole, 20 mM HEPEs, 200 mM KC1, 2 mM β-mercaptoethanol, 0.6 mM ATP, 2 mM MgCl2, and 10% v/v glycerol, pH 7) to remove unbound protein. MgATP was added to this buffer to remove any bacterial DnaK bound to the recombinant CspS. His6-tagged CspS were eluted by application of a gradient of 50–500 mM imidazole, and peak fractions containing the recombinant protein were identified by SDS-polyacrylamide gel electrophoresis. All chromatography was performed at 4 °C using Pharmacia fast protein liquid chromatography system. Hsc70 was purified from bovine brain based on the protocol of Schlossmann et al. (26). The final purified fractions were analyzed by SDS-PAGE, and peak fractions containing Hsc70 were combined. EDTA was added to a final concentration of 4 mM to the purified Hsc70 and mixed at 4 °C for 15 min to restore nucleotide dependent ATPase activity. The purified Hsc70 was dialyzed over night against 20 mM HEPEs-NaOH, 2 mM magnesium acetate, 25 mM KC1, 10 mM NH4SO4, 0.8 mM dTT, pH 7.0, aliquoted, and stored at −80 °C.

ATPase Assays—ATPase assays were performed in a buffer containing 10 mM HEPEs, 100 mM KC1, 2 mM MgCl2, 0.6 mM ATP, 0.5 mM dTT, pH 7. Assays were performed at 37 °C for 1 h, and activity was determined by assaying the release of inorganic phosphate using the spectrophotometric method of Lanpetta et al. (27) with modifications (28). Corrections were made for non-enzymic breakdown of ATP by running duplicate assays on ice and subtracting these from the values obtained at 37 °C. Samples were read at OD280, and their values were compared with a calibration curve using KH2PO4 as a standard.

Tryptic Digestion of Hsc70—Tryptic digests were performed as described previously (29). 4 μg of purified Hsc70 was digested with 0.12 μg of trypsin at 25 °C for 30 min. The digestion was performed in buffer A with 2 mM MgCl2. The digestion products were analyzed by SDS-PAGE on a 12.5% polyacrylamide gel and compared with non-digested Hsc70.

Luciferase Aggregation Assays—Luciferase (1 μg) was added to tubes containing Csp1 (4 μg), Csp2 (4 μg), or Hsc70 (2 μg) in buffer A with 2 mM MgATP. The tubes were incubated at room temperature for 5 min and then transferred to a 42 °C water bath for 10 min. Centrifugation at 16,000 × g for 10 min (4 °C) pelleted aggregated luciferase, which was resuspended in SDS-dissociation buffer, boiled for 5 min, and analyzed on 12.5% polyacrylamide gels. Protein bands were visualized by Coomassie Blue staining. Aggregated luciferase was quantified by densitometry. To examine further the effects of Csp1 and Csp2 on luciferase aggregation, digestion (1 μg) was performed (1 μg) in buffer A with 2 mM MgATP or buffer B (25 mM Tris, 300 mM NaCl, pH 7.8) and 500 μM of buffer B + 50 mM imidazole. The Csp bound to the NTA-NTA beads were eluted with 500 μl of buffer B + 50 mM imidazole, and the eluted proteins were precipitated with an equal volume of methanol at −80 °C and centrifuged at 16,000 × g for 10 min. The pelleted Csp (and associated protein) was resuspended in 50 μl of SDS-dissociation buffer and separated on a 15% polyacrylamide gel. Protein bands were visualized by silver staining or by transfer to nitrocellulose and immunoblotting with specific antisera raised against Hsc70 (Sigma, UK) or luciferase (Europa Ltd., UK). Antibody binding was detected using enhanced chemiluminescence (ECL, Amersham Int.).

RESULTS

One of the aims of this work was to determine the importance of the HPD motif of the J domain of CspS for interaction with Hsc70. Other work had suggested that the conserved HPD motif of J domains is important in the interaction between bacterial and yeast DnaJ homologues. We were interested in whether or not this motif was also involved in the interaction of the synaptic vesicle protein, Csp with Hsc70. To test this, site-directed mutants were generated which were mutated in one of two residues (histidine or aspartic acid) of the conserved HPD motif, and these mutations are depicted in Fig. 1A. The histidine residue was mutated to glutamine (H43Q)
Fig. 1. Stimulation of Hsc70 ATPase activity by Csp1 and effect of mutations in the HPD motif. A, schematic diagram of Csp1 and Csp2 showing the mutations introduced into Csp1; B, dose-response of Csp1 stimulation of Hsc70 ATPase activity. Various concentrations of Csp1 were incubated with Hsc70 (0.4 μM), and release of free phosphate was determined. The inset shows the data plotted as a Hill plot; C, comparison of ATPase stimulation of Hsc70 by Csp1, H43Q, and D45A. Various concentrations of Csp1 (triangles), H43Q (diamonds), and D45A (squares) were incubated with Hsc70 (0.4 μM), and release of free phosphate was determined. Experiments were performed in ATPase assay buffer (see “Experimental Procedures”), and release of free phosphate was measured by a spectrophotometric method (OD660). The levels of phosphate released for each condition were converted into nanomoles of free phosphate liberated by comparison with a KH2PO4 standard after subtraction of any values for Csp alone. Data are expressed as means ± S.E. (n = 4).

Partial tryptic digestion has been used previously to analyze the conformation of DnaK under different conditions (29). A similar protocol was used in this study to examine the effects of ATP and Csp1 on the sensitivity of Hsc70 to tryptic digestion. Fig. 3A demonstrates that the presence of ATP had no significant effect on the sensitivity of Hsc70 to tryptic digestion. However, the presence of Csp1 caused substantially more digestion of Hsc70 (arrowhead), and this was enhanced when ATP was present, suggesting that interaction of Csp1 with Hsc70 to stimulate ATP hydrolysis increases the sensitivity of Hsc70 to tryptic digestion, presumably by a Csp1-induced change in the conformation of Hsc70.

Fig. 2. Effect of H43Q and D45A on ATPase stimulation of Hsc70 by Csp1. Csp1 (0.9 μM) was incubated with Hsc70 (0.4 μM) alone or in the presence of 4.5 μM H43Q or D45A. The control (cont) bar indicates the ATPase activity of Hsc70 alone. The experiment was performed in ATPase assay buffer (see “Experimental Procedures”), and release of free phosphate was measured by a spectrophotometric method (OD660). Data are expressed as means ± S.E. (n = 4).
of the competition experiments, suggesting that the mutants are defective in binding to Hsc70.

By having defined the region of Csp required for binding to Hsc70, we proceeded to analyze the interaction of Csp with substrate proteins. Heat-denatured firefly luciferase is often used as a model substrate for chaperone proteins, and this protein was used in the present study. As an initial approach to examining these interactions, the ability of denatured luciferase to prevent aggregation when present in combination. Interestingly, Csp1 appeared to be slightly better at this function than Csp2, despite the fact that the two proteins had identical ATPase stimulating activity (Ref. 14 and data not shown). At higher concentrations, Csp1 and Csp2 were able to act as molecular chaperones in the absence of Hsc70 by significantly reducing luciferase aggregation (Fig. 5B), and again Csp1 appeared to be slightly more active on a molar basis in this assay. The mutant proteins H43Q and D45A were also effective at preventing aggregation of denatured luciferase at the single concentration tested (Fig. 5B). In contrast, bovine serum albumin had very little effect on luciferase aggregation, confirming that the ability to prevent aggregation is not a general property of all proteins.

If Csps interact with Hsc70 and substrate proteins then it should be possible to isolate complexes of these proteins. Csp was incubated with either luciferase, Hsc70, or both at 42 °C for 10 min, and His6-tagged Csp was isolated by incubation with Ni2+-NTA agarose. Proteins bound to Csp were analyzed by SDS-PAGE and silver staining. Fig. 6A shows that denatured luciferase and Hsc70 each bound to both Csp1 and Csp2 and also that all three proteins could be isolated in a complex. The arrowheads indicate dimer forms of Csp1 and Csp2, the formation of which is well documented (5, 9, 14). Neither luciferase nor Hsc70 bound to Ni2+-NTA agarose in the absence of Csps (Fig. 6B). In addition, it was found that formation of a complex containing Csp, Hsc70, and luciferase was independent of the presence of ATP (Fig. 6B). Complex formation was also analyzed between the mutant proteins H43Q and D45A and Hsc70 and denatured luciferase. Fig. 6C shows that H43Q and D45A bound denatured luciferase to a similar extent as wild type Csp1; however, the mutant proteins were defective in binding Hsc70, consistent with their lack of effect on Hsc70 proteolysis (Fig. 3B).

**DISCUSSION**

The results presented here show that the HPD motif of Csps is important for interaction with Hsc70 and in addition dem-

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**FIG. 4. Effect of Csp1 and denatured luciferase on ATPase activity of Hsc70.** Different combinations of Csp1, luciferase, and Hsc70 (all 0.4 μM) were incubated in ATPase assay buffer at 42 °C, and release of free phosphate was measured by a spectrophotometric method (OD660). The levels of phosphate released for each condition were converted into nanomoles of free phosphate liberated by comparison with a KH2PO4 standard. Data are expressed as means ± S.E. (n = 6).

**FIG. 3. Effects of ATP, Csp1, and mutant proteins on tryptic digestion of Hsc70.** A, Hsc70 (4 μg) was incubated in the presence of different combinations of ATP (2 mM), ATPγS (2 mM), and Csp1 (2.55 μg). B, Hsc70 (4 μg) was incubated in the presence/absence of 2 mM ATP, Csp1, H43Q, and D45A (all 2.55 μg) as indicated. The trypptic digestions were performed in buffer A (with 2 mM MgCl2) with 0.12 μg of trypsin at 25 °C for 30 min. The digestion products were analyzed on a 12.5% polyacrylamide gel stained with Coomassie Blue. The first track shows molecular weight standards, and the second is Hsc70 in the absence of trypsin. All other tracks have Hsc70 digested with trypsin in the absence or presence of ATP or Csps as indicated. The arrowhead indicates the major digestion product in the presence of Csp1. Csps were themselves digested by the trypsin and are not visible.
onstrate that Csp1 and Csp2 can themselves bind denatured protein substrates. The ability of Csps to prevent aggregation of a denatured model protein shows that they can act as chaperones.

Mutation of the histidine residue of the HPD motif to glutamine in E. coli DnaJ and the yeast DnaJ-like protein, Ydj1, abolishes the ability of these proteins to stimulate the ATPase activity of DnaK and Ssa1, respectively (19, 20). Nevertheless, both DnaJ and Ydj1 mutant proteins are still able to interact with their Hsp70 partner proteins but with greatly reduced affinity (20, 32). In this study it was found that the identical mutation in Csp1 reduced the ATPase stimulating activity.

Competition assays, tryptic digestion analyses, and direct binding analyses suggested that this was due to the H43Q mutation extensively reducing the affinity of binding of Csp to Hsc70.

The effect of mutations on the aspartic acid residue (D) of the FIG. 5. Effect of Csps and Hsc70 on luciferase aggregation. A, luciferase (1 μM) was incubated alone or in the presence of Csp1 (4 μM), Csp2 (4 μM), or Hsc70 (2 μM) or combinations of these proteins (n = 8). B, luciferase (1 μM) was incubated in the presence or absence of various concentrations of Csp1, Csp2, and bovine serum albumin or with H43Q or D45A (6 μM) (n = 3). Aggregation assays were performed in buffer A with 2 mM MgATP. Proteins were initially incubated for 5 min at room temperature and then at 42 °C for 10 min to denature luciferase. Aggregated luciferase was pelleted by centrifugation at 16,000 × g for 10 min, resuspended in SDS-dissociation buffer, and analyzed by SDS-PAGE on a 12.5% gel. Aggregated luciferase was quantified by densitometry.

FIG. 6. Analysis of binding of Hsc70 and denatured luciferase to Csp1, Csp2, and Csp mutants. Csp1, Csp2, or Csp mutants (4 μM) were incubated with either Hsc70 (2 μM) or luciferase (1 μM) or both proteins in buffer A with 2 mM MgATP at room temperature for 5 min. The proteins were then incubated at 42 °C for 10 min and centrifuged at 16,000 × g for 10 min to remove large aggregates of luciferase (where appropriate). 50 μl of Ni2+-NTA agarose beads was then added to the proteins and incubated for 30 min on ice. The pellets were then washed in buffer B (see “Experimental Procedures”), buffer B with 50 mM imidazole, and buffer B with 500 mM imidazole. The eluant from the last wash was kept and precipitated with an equal volume of methanol at −80 °C for 20 min. The precipitated proteins were resuspended in SDS-dissociation buffer and separated by SDS-PAGE on a 15% gel. A, binding of Hsc70 and denatured luciferase to Csp1 and Csp2 visualized by silver staining. Arrowheads indicate dimer forms of Csp1 and Csp2; B, effect of ATP on Hsc70 and denatured luciferase binding to Csp1 and Csp2 and lack of binding to control beads in the absence of Csp. Hsc70 and luciferase were detected using specific antisera; C, comparison of Hsc70 and denatured luciferase binding to Csp1 and Csp mutant proteins detected using specific antisera.
HPD motif of DnaJ-like proteins has not been studied previously in any detail. It was found that the D45A mutation virtually abolished the ATPase stimulating activity, and this protein did not bind to Hsc70. This finding, in addition to the results with the H43Q mutant protein, implies that the HPD motif of Csp is important for binding to Hsc70. However, we are not able to determine if this motif is also required for ATPase activation or if this activity is conferred by a separate region of the J domain.

The importance of the HPD motif in DnaJ/Hsp70 interactions is further highlighted as it occurs on a loop region (33–35) and does not have an obvious structural role in the J domain. However, it is also possible that other non-conserved regions of the J domain confer specificity to DnaJ/Hsp70 pairing. Indeed, J domains from the cytosolic DnaJ-like protein, Sis1p, and the mitochondrial protein, Mdj1p, were not functional when substituted into the endoplasmic reticulum DnaJ-like protein Sec63 (36). It was suggested that these hybrid proteins could not interact with Kar2p (an endoplasmic reticulum Hsp70 homologue) and that other non-conserved residues outside the HPD motif are involved in the interaction between Sec63 and Kar2p and other DnaJ/Hsp70 protein pairs.

Partial digestion of DnaK with trypsin reveals that the bacterial protein undergoes a conformational change in the presence of ATP or DnaJ (19, 29). Using a similar protocol we have shown that Hsc70 is more susceptible to tryptic digestion in the presence of Csp1, suggesting that binding of Csp to Hsc70 causes a conformational change in the chaperone protein. The digestion of Hsc70 is further enhanced when both Csp and hydrolyzable ATP are present, implying that binding of Csp to Hsc70 converts Hsc70 into a more “open” conformation and that this is enhanced in the presence of hydrolyzable ATP. The Csp-mediated stimulation of ATP hydrolysis by Hsc70 may be important in enhancing the conformational change of Hsc70 or it may simply allow Csp to cycle on and off Hsc70 resulting in a conformational change in more Hsc70 molecules per unit time than in the absence of ATP. The conformational change of Hsc70 induced by Csp could be important to allow binding to substrate proteins (17).

It has been suggested that the C-terminal region of DnaJ is involved in binding to substrate proteins (37). Since Csp1 and Csp2 differ in the C terminus, their binding to denatured firefly luciferase was examined. We demonstrated that both Csp1 and Csp2 can function as molecular chaperones by preventing the aggregation of denatured luciferase. Since both isoforms are functional in this assay, this suggests that the C-terminal 31 amino acids of Csp are not essential for substrate interaction. Aggregation was almost completely inhibited at a 15-fold molar excess of Csp above luciferase. In comparison, a 50% reduction in aggregation of denatured luciferase was produced in the presence of a 2-fold molar excess of E. coli DnaJ (38). Csp2 also cooperated with Hsc70 to prevent aggregation.

Complexes containing Csp, Hsc70, and luciferase were isolated both in the presence and absence of hydrolyzable ATP. These are likely to be ternary complexes since luciferase did not compete with Csp for Hsc70 ATPase activation implying distinct binding sites, but the presence of binary complexes cannot be excluded. It has been suggested that stimulation of Hsc70 ATP hydrolysis by DnaJ converts Hsc70 into a conformation that can bind to substrate protein (39). However, in this work we found that complex formation is independent of ATP (Fig. 6B). This is in contrast to other work (40) which showed that in the presence of ATP, Hsp1a and Hsp1b (DnaJ-like proteins) stimulated release of the substrate protein carboxymethylated α-lactalbumin from Hsc70. These different results may be due to the choice of model substrate proteins used. However, the stability of the complex between Csp, Hsc70, and luciferase suggests that other protein factors, such as the Hop protein (41), may be required for the full chaperone cycle in vivo.

It was reported that the ability of bacterial DnaJ to bind to denatured protein substrates was dependent on a zinc finger domain (37). However, Csp does not have a recognizable zinc finger domain, yet can bind directly to a substrate protein. It may be that some other region of Csp can substitute for a zinc finger domain in specifying substrate interaction or that Csp has a non-consensus zinc-binding site. Since Csp1 and Csp2 are both functional as molecular chaperones, the question arises as to the function of the C-terminal 31 amino acids of Csp1. One possibility is that the C terminus of Csp may determine the spectrum of possible substrate interactions in the synapse. This question is clearly important in light of the finding that Csp1 and Csp2 show distinct developmental and adult profiles of expression in rat brain (16).

The finding that Csp can bind to an unfolded protein substrate has implications for the function of Csp in regulated exocytosis. Csp may interact with synaptic proteins that are in a partially unfolded or an energetically unfavorable conformation. This could be a potentially important interaction in exocytosis as it is well established that the docking and fusion of synaptic vesicles to presynaptic membranes involve numerous protein-protein interactions. For example, it has been proposed that the actions of the synaptic proteins N-ethylmaleimide-sensitive factor and soluble N-ethylmaleimide-sensitive factor attachment proteins is to modify the conformation of SNARE proteins on the vesicle or target membrane to allow them to interact (42). The interaction of Csp with unfolded proteins may explain why there has been little success in identifying proteins that bind Csp (14). These data suggest two new possibilities for Csp function in exocytosis as follows: first, Csp interacts with the unfolded conformational state of a particular protein, and second, Csp is a general secretory vesicle chaperone, functioning by binding to multiple vesicle proteins in their unfolded conformations. Finally, it is also possible that Csp may have a more specific function in regulated exocytosis, in addition to the proposed general chaperone role. Further work will be required to resolve this issue.

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REFERENCES

Cysteine String Protein Interactions
