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Short Review

Cysteine-String Protein: The Chaperone at the Synapse

Luke H. Chamberlain and *Robert D. Burgoyne

*Division of Biochemistry and Molecular Biology, University of Glasgow, Glasgow, Scotland; and *The Physiological Laboratory, University of Liverpool, Liverpool, England*

Abstract: Cysteine-string protein (Csp) is a major synaptic vesicle and secretory granule protein first discovered in *Drosophila* and *Torpedo.* Csps were subsequently identified from *Xenopus, Caenorhabditis elegans,* and mammalian species. It is clear from the study of a null mutant in *Drosophila* that Csp is required for viability of the organism and that it has a key role in neurotransmitter release. In addition, other studies have directly implicated Csp in regulated exocytosis in mammalian neuroendocrine and endocrine cell types, and its distribution suggests a general role in regulated exocytosis. An early hypothesis was that Csp functioned in the control of voltage-gated Ca^{2+} channels. Csp, however, must have an additional function as a direct regulator of the exocytotic machinery as changes in Csp expression modify the extent of exocytosis triggered directly by Ca^{2+} in permeabilised cells. Csps possess a cysteine-string domain that is highly palmitoylated and confers membrane targeting. In addition, Csps have a conserved "J" domain that mediates binding to an activation of the Hsp70/Hsc70 chaperone ATPases. This and other evidence implicate Csps as molecular chaperones in the synapse that are likely to control the correct conformational folding of one or more components of the vesicular exocytotic machinery. Targets for Csp include the vesicle protein VAMP/synaptobrevin and the plasma membrane protein syntaxin 1, the significance of which is discussed in possible models to account for current knowledge of Csp function. **Key Words:** Synaptic vesicle—Secretory granule—Secretion—Neurotransmission—Exocytosis—SNAREs.
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The release of neurotransmitters from nerve endings is a highly complex and tightly regulated process involving the specific and sequential interactions of a vast array of neuronal proteins. Despite the complexity of the exocytotic machinery, neurotransmitter secretion can occur within <100 μs of nerve terminal depolarisation and Ca^{2+} entry (Burgoyne and Morgan, 1995). Combined biochemical and genetic analysis of synaptic proteins has allowed a detailed molecular description of presynaptic neurotransmission to begin to emerge (Südhof, 1995; Calakos and Scheller, 1996; Hanson et al., 1997; Fernandez-Chacon and Südhof, 1999). Recent attention has been focused on the role of the so-called soluble (*N*-ethylmaleimide-sensitive factor)-attachment protein receptor (SNARE) proteins [vesicle-associated membrane protein (VAMP)/synaptobrevin, syntaxin 1, and SNAP-25] as a vesicle docking or fusion machinery (Sollner et al., 1993; Sutton et al., 1998; Weber et al., 1998). The SNARE proteins are able to form a stable complex both in vivo and in vitro (Hayashi et al., 1994), and a number of proteins appear to regulate the interactions of the SNAREs and their ability to form a complex. Although many presynaptic proteins have now been identified, much still remains to be done to establish their exact functions. The synaptic vesicle protein cysteine-string protein (Csp) is an essential component of the neurotransmitter release machinery. Until recently, however, the function of Csp in neurotransmission was unclear. Here we discuss the possible role of Csp in presynaptic neurotransmission, focusing on recent work that has suggested that Csp may perform a chaperone function in the synapse, with the synaptic vesicle protein VAMP/synaptobrevin and the plasma membrane protein syntaxin 1 as possible substrates. This recent work implicates Csp in the regulation of SNARE protein interactions.

IDENTIFICATION OF Csps AND THEIR PROTEIN SEQUENCES

Csps were first identified as neuronal-specific antigens recognised by a monoclonal antibody raised against *Drosophila* heads (Zinsmaier et al., 1990), and later as potential Ca^{2+}-channel regulators in *Torpedo* (Gundersen and Umbach, 1992). Csps from *Drosophila* and *Torpedo* were detected originally in brain and highly innervated

Address correspondence and reprint requests to Dr. R. D. Burgoyne at The Physiological Laboratory, University of Liverpool, Crown Street, Liverpool L69 3BX, U.K. E-mail: burgoyne@liverpool.ac.uk

Abbreviations used: Csp, cysteine-string protein; Hsc70, 70-kDa heat-shock cognate protein; Hsp70, heat-shock protein of 70 kDa; SNARE, soluble (*N*-ethylmaleimide-sensitive factor)-attachment protein receptor; VAMP, vesicle-associated membrane protein.

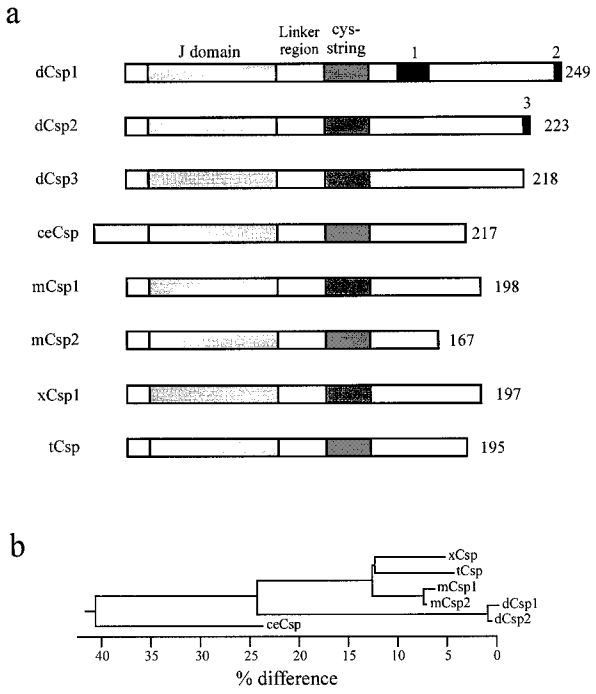

FIG. 1. a: Schematic representation of the Csps showing the position of the conserved J domain and cysteine string. Csps from various species are shown schematically, including three splice variants from *Drosophila* (dCsp1, dCsp2, dCsp3), *Caenorhabditis elegans* (ceCsp), two mammalian (bovine, human, and rat) splice variants (mCsp1, mCsp2), and Csps from *Xenopus* (xCsp1) and *Torpedo* (tCsp). The black regions in the *Drosophila* Csps show the alternatively spliced domains 1, 2, and 3. **b:** Relationships between Csp from various species shown in the form of a phylogenetic tree.

tissues, but not in nonneuronal cells (Zinsmaier et al., 1990; Umbach and Gundersen, 1991; Mastrogiacomo et al., 1994*a*). It has been demonstrated subsequently that the expression of Csp is not neuronal-specific as discussed below. Two Csp isoforms have since been identified in mammals (Braun and Scheller, 1995; Mastrogiacomo and Gundersen, 1995; Chamberlain and Burgoyne, 1996; Coppola and Gundersen, 1996): Csp1 is the mammalian homologue of the *Torpedo* protein, whereas Csp2 is a novel C-terminally truncated isoform (see Fig. 1a). Csp2 mRNA is generated by alternative splicing (Kwon et al., 1996) and is present in a number of tissues, but expression of the Csp2 protein has been difficult to demonstrate convincingly. In *Drosophila*, three alternative splice isoforms are known (Zinsmaier et al., 1990). Csps have also been identified in *Xenopus* (Mastrogiacomo et al., 1998*b*) and *Caenorhabditis elegans* (GenBank accession number U40415). The *C. elegans* Csp is the most distant in terms of sequence similarity, having (Fig. 1b) no more than ~35% overall identity with Csp from any other species. The *Drosophila* Csps are 50–60% identical to the mammalian Csps, which are themselves 98–100% identical.

Analysis of the protein sequence of Csps reveals two distinctive domains (Fig. 1): a central cysteine-rich region containing a high density of cysteine residues (14 cysteines in a span of 24 amino acids for *Torpedo* and mammalian Csps), and an N-terminal "J" domain that is highly homologous (50% identity; Fig. 2) to a region of the well characterised bacterial chaperone protein DnaJ (Caplan et al., 1993; Silver and Way, 1993). Within Csps, the J domain is highly conserved with at least 78% identity between species. A number of eukaryotic proteins have J domains, and these proteins have been shown to interact with and regulate members of the Hsp70 (heat-shock protein of 70 kDa) chaperone family by virtue of this domain (Cheetham and Caplan, 1998; Kelley, 1998). The J domains of various mammalian proteins have from 26 to 60% identity with that of mammalian Csp, with the J domain most similar to that in Csp being in the human protein HSJ2 (Fig. 2). All of the known J domain proteins have an absolutely conserved HPD motif required for interaction with Hsp70 proteins (Bukau and Horwich, 1998) as discussed below.

Csps also have a highly conserved "linker" region of ~20 amino acids that links the J and cysteine-string domains (Fig. 3). The function of the linker region is not known, but it has been shown to be important for the function of Csps in exocytosis (Zhang et al., 1999). Whereas the J domain, cysteine-string region, and linker region are highly conserved, the C-terminal domains of Csps are more variable and alternative splicing in this region generates isoforms in *Drosophila* and in mammalian species. The domain formed by insert 1 in *Drosophila* Csp1 (Fig. 1) and the 40–50 amino acids C-terminal to this have no homology with the C-terminus of Csps in other species.

GENETIC STUDIES OF Csp FUNCTION IN *Drosophila*

The involvement of Csp in presynaptic neurotransmission became apparent with the generation and characterisation of *Drosophila* Csp null mutants (Umbach et al.,

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          *  **  * *    * * * *** ***** *    **   *****  *   ***  **  ** **
DnaJ   5 DYYEILGVSKTAEEREIRKAYKRLAMKYHPDRNQGDKEAEA-KFKEIKEAYEVLTDSQKRAAYDQYGHAAF 74
mCsp1  5 SLYHVLGLDKNATSDDIKKSYRKLALKYHPDKNPDNPEAAD-KFKEINNAHAILTDATKRNIYDKYGSLGL 84
HSJ2   3 DYYEVLGVQRHASPEDIKKAYRKLALKWHPDKNPENKEEAERKFKQVAEAYEVLSDAKKRDIYDKYGKEGL 73
           * ***    *   **** ******* ****** * * *  ***    *   * ** ** ******  **
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FIG. 2. Comparison of the J domains of Csp1, HSJ2, and DnaJ. The sequence within the J domain of the mammalian Csp1 is aligned with that of the *E. coli* protein DnaJ and the human protein HSJ2, which is the mammalian protein with the most similar J domain sequence. Residues identical in DnaJ and Csp1 are indicated by asterisks above the alignments, and those identical in HSJ2 and Csp1 are indicated below.

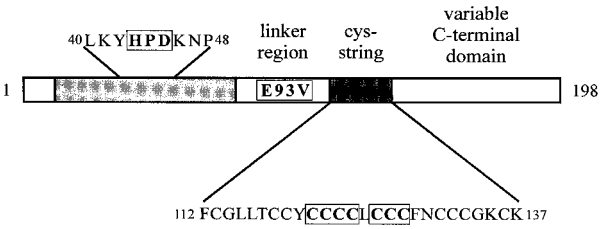

FIG. 3. Key domains of Csp and effects of mutagenesis. The bovine Csp1 is shown schematically to indicate the various domains of the protein and key residues that have been analysed functionally by mutagenesis of this protein. The conserved HPD motif of the J domain has been shown to be essential for binding of Csp to Hsc70 and activation of its ATPase activity (Chamberlain and Burgoyne, 1997*b*). Within the cysteine string, mutation of seven of the cysteines to serines (boxed) abolishes palmitoylation and membrane targeting (Chamberlain and Burgoyne, 1998*b*). Mutation of the conserved glutamate 93 in the linker region to valine disrupts Csp interactions with the exocytotic machinery (Zhang et al., 1999).

1994; Zinsmaier et al., 1994). Inactivation of the Csp gene had a profound effect on *Drosophila* viability, with only 4% of the mutant flies surviving into adulthood, and even these were abnormal (Zinsmaier et al., 1994). Analysis of the surviving flies showed that they had a paralytic phenotype, displaying uncoordinated, sluggish movements, spasmic jumping, and intense shaking. Furthermore, the surviving adult mutant flies had a severely reduced lifespan and died within 4–5 days at 22°C, or within 1 h if the temperature was raised to 30°C. This initial observational characterisation of *Drosophila* Csp null mutants demonstrated that Csps are essential proteins for *Drosophila* viability.

The phenotypical abnormalities exhibited by the Csp mutant flies suggested that Csp may have a role in neurotransmission. This idea was confirmed by electrophysiological analysis of Csp mutant larvae, which revealed a defect in evoked transmitter release at their neuromuscular junctions (Umbach et al., 1994). Postsynaptic recordings showed that depolarisation-induced neurotransmitter exocytosis was decreased by ~50% at 22°C in the mutant embryos (Umbach et al., 1994), and the remaining release was no longer synchronous (Heckmann et al., 1997), indicating a major role for Csp in neurotransmission. In addition to this major defect, neurotransmission failed completely when the temperature was raised to 30°C. This is not a temperature-sensitive mutation as Csp is not expressed, but this additional temperature-sensitive phenotype suggests that components of the neurotransmitter release machinery are unstable at elevated temperature in the absence of Csps and implies that Csp normally has a protective role at higher temperatures. Spontaneous release was also decreased in the mutant larvae, but this deficiency was not as pronounced as that of evoked release, and it is interesting that there was no temperature-sensitive effect on spontaneous release from Csp null mutants (Umbach et al., 1994). There appears to be redundancy between the three *Drosophila* Csp isoforms, and all three are individually sufficient to rescue the null mutant (Nie et al., 1999).

Although this work clearly demonstrated a requirement for Csps in presynaptic neurotransmission, it was not known whether Csps acted in the exocytosis or recycling of synaptic vesicles. Indeed, it was suggested that because Hsc70 (70-kDa heat-shock cognate protein), the constitutively expressed form of Hsp70, is involved in clathrin uncoating (Chappell et al., 1986), then Csp may also function in a synaptic vesicle endocytosis (Südhof, 1995). However, a more detailed analysis of the mutant larvae, using FM-143 (Ranjan et al., 1998), demonstrated that the internalisation of this lipophilic dye into synaptic terminals after exocytosis was normal, implying that vesicle retrieval is not affected by the absence of Csp. In contrast, depolarisation-induced release of FM-143 from loaded terminals was reduced at permissive temperatures and almost completely inhibited at higher temperatures from mutant preparations. Nevertheless, nerve terminals could still be destained by black widow spider venom, which stimulates depolarisation-independent release, and exocytosis could also be evoked from mutant terminals by Ca^{2+} ionophores such as ionomycin (Umbach and Gundersen, 1997). These analyses of Csp mutant synapses demonstrated that vesicles are intact and releasable in Csp null mutants and can undergo endocytosis, but that there is some defect in excitation–secretion coupling. These results are therefore consistent with a role for Csps in the steps leading to exocytosis rather than in vesicle retrieval.

The studies on *Drosophila* Csps are complemented by work on PC12 cells and pancreatic β cells. Such cell types are often used as model systems of regulated exocytosis (Burgoyne and Morgan, 1998; Lang, 1999) and have also contributed to our understanding of presynaptic neurotransmission. Stable overexpression of Csp in PC12 cells specifically increased the extent of Ca^{2+}-stimulated exocytosis without affecting other aspects of the cells (Chamberlain and Burgoyne, 1998*a*), whereas reduction of Csp levels by an antisense strategy decreased regulated exocytosis from an insulin-secreting cell line (Zhang et al., 1998). Therefore, like the majority of proteins involved in presynaptic neurotransmission, Csp is also involved in regulated exocytosis from other cell types. These studies are also important as they demonstrate that Csps function in regulated exocytosis in mammalian cells in addition to *Drosophila.* Also, other work based on injection of anti-Csp antibodies into the neuromuscular junction of *Xenopus* in nerve–muscle cultures (Poage et al., 1999) showed inhibition of neurotransmission, demonstrating an acute requirement for Csp in synaptic exocytosis. Surprisingly, transient overexpression of Csp in insulin-secreting cells was also inhibitory (Brown et al., 1998; Zhang et al., 1999), and recently Csp overexpression in vivo in *Drosophila* was also found to be deleterious (Nie et al., 1999). These findings may seem paradoxical, but a similar deleterious effect of syntaxin overexpression has been reported (Wu et al., 1998) despite this protein being essential for exo-

cytosis (Broadie et al., 1995). The use of this transient overexpression strategy has allowed an examination of key residues in Csp that affect exocytosis and pinpointed the importance of the conserved linker region (Zhang et al., 1999). In addition, transient overexpression of Csp1 in adrenal chromaffin cells also reduced exocytosis, but more significantly modified the kinetics of single granule release events from permeabilised cells monitored by amperometry (Graham and Burgoyne, 2000). These results suggest that Csp exerts its effects at the level of the machinery comprising the fusion pore or that regulates fusion-pore expansion.

TISSUE DISTRIBUTION AND SUBCELLULAR LOCALISATION OF Csps

Early work on Csps suggested that the proteins were present only in neuronal cells (Zinsmaier et al., 1990; Umbach and Gundersen, 1991; Mastrogiacomo et al., 1994*a*). However, it is now well established that although Csps are enriched in neurons, they are also expressed in various nonneuronal cell types (Kohan et al., 1995; Chamberlain and Burgoyne, 1996; Coppola and Gundersen, 1996; Eberle et al., 1998). Csps are associated with synaptic vesicles in neurons, where they account for 1% of total vesicle protein, with about eight Csp monomers per vesicle (Mastrogiacomo et al., 1994*b*; Van de Goor et al., 1995). Csps are also present on secretory granules in nonneuronal cells, including endocrine, neuroendocrine, and exocrine cell types (Braun and Scheller, 1995; Chamberlain et al., 1996; Pupier et al., 1997; Brown et al., 1998; Zhang et al., 1998). They are abundantly expressed in nonneuronal cells with a high secretory capacity, making up 0.45% of total adrenal chromaffin cell protein, for example (Chamberlain et al., 1996).

Although it is clear that Csps function in presynaptic neurotransmission and other regulated exocytotic pathways, it is not known whether Csps also have a role in the exocytosis of constitutive secretory vesicles, although it has been demonstrated that spontaneous neurotransmitter release persists in the *Drosophila* null mutant (Umbach et al., 1994). Expression of Csps has been demonstrated in a wide range of tissues not normally associated with regulated exocytosis, including liver, kidney, spleen, and muscle (Chamberlain and Burgoyne, 1996; Eberle et al., 1998). Recent work, however, has suggested that Ca^{2+}-regulated exocytosis is ubiquitous (Coorssen et al., 1996; Ninomiya et al., 1996) possibly due to Ca^{2+}-dependent exocytosis of lysosomes (Rodriguez et al., 1997). The role of Csp in this form of exocytosis in so-called nonregulated secretory tissues remains to be established. It should be noted that there is no obvious *Saccharomyces cerevisiae* homologue of Csp, and so it is likely that Csps do not act at early stages of the secretory pathway or in constitutive exocytosis and that their function is restricted to regulated exocytotic membrane fusion events that do not occur in yeast. Alternatively, there may be other DnaJ-like proteins that have analogous activities to Csp and operate at other steps of the secretory pathway.

THE CYSTEINE-STRING DOMAIN IN VESICLE TARGETING, ATTACHMENT, AND FUSION

The most striking feature of Csps, which gives rise to their name, is the cysteine-string domain containing a large number of closely spaced cysteine residues. The palmitoylation of these cysteine residues combined with the charged N- and C-termini of Csp gives the protein an amphipathic structure. These structural features of Csps led to speculation that Csp may be able to interact with membrane lipids in such a way as to bring about the fusion of membranes in close apposition (Gundersen et al., 1995). Although this is an interesting idea, it is unlikely to be correct as there is no yeast homologue of Csp, and yet it is well established that the fundamental exocytotic machinery for membrane fusion is conserved between yeast and higher eukaryotes (Bennett and Scheller, 1993). It is also unlikely that the deletion of an essential membrane fusogen could be compensated for in the few surviving Csp null mutant *Drosophila* or that mutant larvae would exhibit only a 50% decrease in evoked release at permissive temperatures.

In contrast to the lack of evidence for Csp acting as a membrane fusogen, there is good evidence that a function of the palmitoylated cysteine-string domain is to mediate the correct membrane association of Csp (Gundersen et al., 1994). Complete chemical depalmitoylation, however, does not displace Csp from *Drosophila* and PC12 cell membranes, suggesting that other nonpalmitoylated regions of Csp also interact with membranes (Van de Goor and Kelly, 1996; Chamberlain and Burgoyne, 1998*b*). Studies on the plasma membrane protein SNAP-25 (Gonzalo and Linder, 1998) showed that palmitoylation is required for initial membrane targeting, but not stable membrane association of this protein. Similarly, mutation of the central cysteine residues in the cysteine-string domain of Csp (see Fig. 3) abolished palmitoylation and membrane association upon transfection into PC12 and HeLa cells (Chamberlain and Burgoyne, 1998*b*). Thus, palmitoylation of the cysteine-string domain of Csp, like SNAP-25 palmitoylation, is required for initial membrane targeting, but not stable membrane association. It has been suggested that it is the hydrophobic cysteine-string region that ensures Csp membrane attachment in the absence of palmitoylation (Mastrogiacomo et al., 1998*a*). This idea is particularly interesting as some of the cysteine residues in the string domain may not be palmitoylated (Gundersen et al., 1994; Chamberlain and Burgoyne, 1998*b*), and it is feasible that these nonpalmitoylated cysteines may be embedded in the membrane lipid. In addition, Csps have a series of hydrophobic residues at the C-terminal side of the linker region and thus next to the cysteine-string domain. It is possible that this polar domain contributes to the membrane association of the Csps. Future studies

may identify regions of Csp that are important for the stable association of Csps with membranes. It will also be particularly interesting to determine the nature of the targeting signals that direct palmitoylated Csp to synaptic vesicles or secretory granules, but palmitoylated SNAP-25 predominantly to the plasma membrane.

Csps AND Ca^{2+}-CHANNEL REGULATION

A suppression cloning study by Gundersen and Umbach (1992) suggested a role for Csp in the regulation of voltage-dependent Ca^{2+} channels. It was found that Csp antisense RNA could inhibit the activity of N-type Ca^{2+} channels from *Torpedo* functionally expressed in *Xenopus* oocytes from brain RNA. Conversely, Csp sense RNA enhanced the activity of the exogenously expressed Ca^{2+} channels. The initial explanation for these findings was that Csp is a subunit of N-type Ca^{2+} channels; however, it was subsequently shown that Csps are primarily components of synaptic vesicles (Mastrogiacomo et al., 1994*b*; Van de Goor et al., 1995), arguing against this idea. This led to the modified suggestion that vesicle-associated Csp may function to increase Ca^{2+}-channel activity at the sites of vesicle docking on the presynaptic membrane, ensuring that Ca^{2+} entry is greatest through channels that are physically linked to vesicles (Mastrogiacomo et al., 1994*b*).

An interaction between Csp and a recombinant loop of the P/Q-type Ca^{2+} channel has been shown in vitro (Leveque et al., 1998), but no direct in vitro association of Csps with N-type Ca^{2+} channels has been observed nor is there any evidence of direct interaction with any Ca^{2+}-channel subtypes in vivo, questioning Ca^{2+}-channel regulation as the sole function of Csps. In addition, the widespread tissue distribution of Csps (Chamberlain and Burgoyne, 1996; Coppola and Gundersen, 1996; Eberle et al., 1998) implies that the major function of Csps in exocytosis is not to regulate Ca^{2+}-channel activity, as a number of the tissues in which Csps are expressed do not apparently contain cells with voltage-gated Ca^{2+} channels. In addition, Csp has been shown to have Ca^{2+} channel-independent functions in exocytosis demonstrable in permeabilised cells in which Ca^{2+} or guanosine 5′-*O*-(3-thiotriphosphate) directly activates exocytosis (Chamberlain and Burgoyne, 1998*a*; Zhang et al., 1998, 1999). Overexpression of Csps in PC12 cells and insulin-secreting cells does not affect depolarisation-induced Ca^{2+} influx, suggesting that Csps do not have any major effects on Ca^{2+}-channel activity in these cells (Brown et al., 1998; Chamberlain and Burgoyne, 1998*a*). In contrast, Csp overexpression altered the exocytotic responses due to direct triggering with Ca^{2+}. Finally, recent results show that Ca^{2+} currents are not affected in peptidergic synapses in Csp null mutants of *Drosophila*, arguing against a general role as Ca^{2+}-channel regulators (Morales et al., 1999).

Some of the aforementioned studies may be limited by the sensitivity of the assays used, and overexpression of Csp may not noticeably affect channel activity if Csp is already expressed at a sufficient level in wild-type cells. It is also possible that Csps may regulate only the activity of Ca^{2+} channels that function in fast neurotransmitter release (N- and P/Q-type channels) and not those that stimulate slower exocytosis from PC12 and pancreatic β cells (typically L-type channels) and peptidergic nerve terminals. In support of this idea, recent work using Ca^{2+}-sensitive fluorescent dyes has shown that depolarisation-induced Ca^{2+} entry into glutaminergic nerve terminals is reduced in *Drosophila* Csp null mutants (Umbach et al., 1998), suggesting some link between Csps and Ca^{2+} channels. A surprising aspect of this report was that a temperature-sensitive mutation in dynamin, a protein involved in endocytosis, also resulted in a loss in Ca^{2+} entry at elevated temperatures subsequent to the depletion of synaptic vesicles. It remains, therefore, to be established whether the effect of Csp expression on Ca^{2+}-channel activity in these nerve terminals is by a direct or indirect mechanism. The data overall strongly suggest that Csps have functions in regulated exocytosis independent of any regulation of Ca^{2+}-channel function possibly due to actions exerted directly on the fusion machinery.

Csps AS MOLECULAR CHAPERONES

Understanding the role of Csps in regulated secretion is perhaps best approached with an appreciation of the activities of other J domain-containing proteins that are members of the DnaJ family (Cheetham and Caplan, 1998; Kelley, 1998), as Csp can be expected to have similar molecular activities. DnaJ proteins are essentially adaptor proteins that recruit the Hsp70 ATPases to substrates specified by the DnaJ family member. At these sites, the ATPase activity of Hsp70 is activated by the DnaJ protein and the energy created by ATP hydrolysis is used to bring about a particular change in protein conformation (Bukau and Horwich, 1998). Established roles for DnaJ/Hsp70 protein pairs are in the folding of nascent polypeptides, the protection and refolding of denatured proteins, the translocation of polypeptides across membranes, and the assembly/disassembly of protein complexes (Hartl, 1996).

One other DnaJ protein, auxilin, is known to be enriched at synaptic sites. Auxilin interacts with clathrin-coated vesicles and functions in conjunction with Hsc70 as the machinery for ATP-dependent uncoating of the vesicles and removal of clathrin coat after vesicle retrieval (Ungewickell et al., 1995). Whereas DnaJ proteins such as auxilin are thought to have specific substrate activities (in this case clathrin), other DnaJ proteins have affinity for particular conformers exhibited by a number of proteins under different conditions. For example, the chaperone activities of DnaJ proteins that act in general protein folding reactions seem to be bestowed by an affinity for unfolded hydrophobic regions of proteins or polypeptides. Binding of the DnaJ proteins to these unfolded structures stabilises them and also mediates refolding/folding when Hsp70 is recruited and the

DnaJ protein stimulates its ATPase activity (Hartl, 1996). The bacterial Hsp70, DnaK, is known to have a preference for binding to a short (five-residue) exposed hydrophobic core flanked by basic residues (Rudiger et al., 1997), but the sequence or structural requirements for binding of specific DnaJ proteins to their substrates are still to be determined.

Csp functions as a typical DnaJ protein as it is able to interact with and activate the ATPase activity of the heat-shock inducible Hsp70 and the constitutively expressed protein Hsc70 (Braun et al., 1996; Chamberlain and Burgoyne, 1997*a*), by virtue of the conserved "HPD" motif (Fig. 3) in its J domain (Chamberlain and Burgoyne, 1997*b*). This interaction involves both the substrate-binding domain and the ATPase domains of Hsc70 and is specific as no binding occurs to Hsp60 or Hsp90 (Stahl et al., 1999). Like a number of other DnaJ proteins, Csps can bind denatured model proteins, such as firefly luciferase, and prevent their aggregation, showing that Csps have affinity for unfolded proteins and can act as general chaperones (Chamberlain and Burgoyne, 1997*b*). Furthermore, Csp and Hsc70 function synergistically to prevent aggregation of denatured proteins (Chamberlain and Burgoyne, 1997*b*), suggesting that the two proteins can function as a chaperone machinery. Similar studies on other DnaJ proteins have indicated that the ability of DnaJ proteins to bind to denatured proteins and recruit Hsp70 proteins may be sufficient to cause reactivation of the denatured protein in the presence of hydrolysable ATP (Terada et al., 1997). Therefore, it is likely that Csp and Hsc70 have a similar "foldase" activity and are able to influence the conformational status of synaptic proteins.

The following points are consistent with the idea that Csp acts as a presynaptic chaperone in neurotransmission: (a) Csp is a member of the well characterised DnaJ family of chaperone proteins; (b) Csp has affinity for unfolded model substrate proteins; (c) Csp interacts with the co-chaperone Hsc70; (d) inactivation of the *csp* gene in *Drosophila* causes a temperature-sensitive phenotype with additionally defective neurotransmission consistent with a protective function for Csp at higher temperatures; and (e) Csp interacts with VAMP (see below), which lacks secondary structure.

SUBSTRATES FOR Csp

Recent work has shown that Csp coimmunoprecipitates with the synaptic vesicle protein VAMP from rat brain membranes (Leveque et al., 1998). This protein is known to play a key role in neurotransmission (Schiavo et al., 1992; Sweeney et al., 1995; Deitcher et al., 1998), and its interaction with the plasma membrane proteins syntaxin and SNAP-25 is essential for membrane fusion (Sollner et al., 1993). The interaction of Csps with VAMP is particularly interesting in light of the finding that Csps have affinity for denatured proteins (Chamberlain and Burgoyne, 1997*b*), as VAMP has been shown to have little secondary structure and, therefore, to have the characteristics of an unfolded protein (Fasshauer et al., 1997). Thus, one role of Csp in presynaptic neurotransmission may be to act as a chaperone for VAMP on the vesicle. The temperature-sensitive phenotype of Csp mutants (Umbach et al., 1994), which suggests loss of a chaperone activity required at elevated temperature, could result from instability of VAMP (or some other protein). The idea that absence of Csp could result in thermal instability of another protein is supported by work showing that deletion of the DnaJ gene in *E. coli* causes a temperature-sensitive growth arrest and instability of DnaJ substrate proteins (Ohki et al., 1992). An interesting comparison, however, is that DnaJ is not essential for cell survival in *E. coli*, whereas Csp absence results in failure to survive into adulthood (except for a few survivors) even at low temperature in *Drosophila* (Zinsmaier et al., 1994), indicating the essential requirement for Csp even in the absence of heat shock.

The possibility that Csp and VAMP may functionally interact in vivo is suggested by the similar phenotypes of Csp and neuronal VAMP null mutants in *Drosophila.* Mutation of either the VAMP or Csp gene eliminates depolarisation-induced but not spontaneous release (Umbach et al., 1994; Yoshihara et al., 1999). In addition, although neurotransmitter secretion is no longer evoked by nerve terminal depolarisation, exocytosis from both mutants can be triggered by Ca^{2+} ionophores (Umbach and Gundersen, 1997; Yoshihara et al., 1999). Further work will be required to determine if these phenotypes are indeed related.

In immunoprecipitation studies on mammalian brain, Csp was not found to interact with syntaxin 1 (Leveque et al., 1998). In contrast, recent work has provided clear evidence of an interaction between *Drosophila* Csp and syntaxin 1A both in vitro (Wu et al., 1999) and, importantly, in vivo (Nie et al., 1999). *Drosophila* Csp could be immunoprecipitated in a complex with syntaxin and the recombinant proteins shown to interact directly (Wu et al., 1999). Syntaxin 1 is essential for neurotransmitter release (Broadie et al., 1995), but its overexpression in *Drosophila* also inhibits neurotransmission (Wu et al., 1999). Recent functional studies have shown that the deleterious effects of syntaxin 1A overexpression could be overcome by co-overexpression of Csp, providing evidence of an interaction of the two proteins in vivo (Nie et al., 1999). These data, therefore, provide evidence of a second substrate for Csp. The reasons for the very different findings from immunoprecipitation from rat brain compared with *Drosophila* are not known, and further work will be required to determine whether or not mammalian Csps can bind syntaxin 1. One important aspect will be the determination of the domain of Csp required for the interaction of *Drosophila* Csp with syntaxin. Does this involve a conserved region of *Drosophila* Csp or the nonconserved C-terminus of *Drosophila* Csp?

MODELS FOR Csp FUNCTION IN REGULATED EXOCYTOSIS

Current data indicate that although Csp may have a role in the regulation of Ca^{2+} channels, it must also have additional direct roles in the exocytotic machinery. The interaction of VAMP with the plasma membrane proteins syntaxin and SNAP-25 forms the very stable SNARE complex (Sutton et al., 1998), which is essential for membrane fusion (Littleton et al., 1998) and is believed to be the core of the conserved vesicle docking and fusion machinery in constitutive exocytosis in yeast and regulated exocytosis in the synapse. A key issue that remains to be understood is why Csp interacts with conserved components of the docking/fusion machinery and yet no Csp homologue exists in yeast. This suggests a specialised role for Csp in linking the SNARE complex with the exocytotic Ca^{2+} sensors such as synaptotagmin with which the SNAREs directly interact (Südhof and Rizo, 1996).

Syntaxin and SNAP-25 are also present on synaptic vesicles (Walch-Solimena et al., 1995), but they are far less abundant than VAMP on these vesicles, and therefore the majority of VAMP molecules on synaptic vesicles are not present in SNARE complexes. Monomeric VAMP has virtually no secondary structure (Fasshauer et al., 1997), and therefore, one possible function for Csp is to act to stabilise VAMP, allowing it to interact efficiently with plasma membrane-associated syntaxin and SNAP-25 to form an active SNARE complex for membrane fusion. The role of Hsc70 may be to disengage the Csp–VAMP complex, allowing efficient SNARE complex assembly, or alternatively, Hsc70 may promote a conformational change in VAMP, increasing its affinity for syntaxin/SNAP-25. As Ca^{2+} ionophores, but not nerve terminal depolarisation, are effective at stimulating transmitter release from Csp and VAMP null mutants (Umbach and Gundersen, 1997; Yoshihara et al., 1999), it may be that vesicles are uncoupled from sites of exocytosis close to Ca^{2+} channels in these mutants.

Syntaxin and SNAP-25 interact with and negatively regulate presynaptic Ca^{2+} channels expressed in model systems (Bezprozvanny et al., 1995; Wiser et al., 1996). The binding of Csp to syntaxin involves the same syntaxin domain that binds N-, P-, and Q-type channels at the cytoplasmic loop between transmembrane domains II and III. This has been called the synprint site (Sheng et al., 1996). It has been suggested that this could explain regulation of voltage-gated Ca^{2+} channels by Csp due to removal of syntaxin from the synprint site (Wu et al., 1999). It is not known whether the interaction of syntaxin with Ca^{2+} channels occurs in a physiological situation where syntaxin has many other binding partners and may not be available for such an interaction. One possibility, however, is that the interaction of Csp with syntaxin removes this inhibitory effect in the *Xenopus* expression system where effects of Csp on Ca^{2+}-channel activity were observed. Thus, the observed effects of Csp inactivation on Ca^{2+}-channel activity (Gundersen and Umbach, 1992) may have resulted indirectly from removal of inhibition by syntaxin. It remains to be seen, however, whether Csp does regulate Ca^{2+}-channel activity in the synapse rather than regulating interactions between SNARE proteins in a fusion complex or modifying vesicle docking into a rapidly releasable pool close to presynaptic Ca^{2+} channels via SNARE proteins. An effect on SNARE interactions is more consistent with work showing that Csp overexpression does not effect Ca^{2+}-channel activity in PC12 cells and pancreatic β cells (Brown et al., 1998; Chamberlain and Burgoyne, 1998*a*) and that Ca^{2+} currents in peptidergic nerve terminals are unaffected by the absence of Csp (Morales et al., 1999), but that Csp has direct effects on Ca^{2+}-triggered exocytosis in permeabilised cells (Chamberlain and Burgoyne, 1998*a*; Zhang et al., 1998, 1999).

The discussion above emphasises the finding that VAMP and syntaxin 1A are so far the only synaptic proteins that have been demonstrated to bind to Csp. It cannot be ruled out, however, that Csp interacts with other synaptic substrates or may even have a more nonspecific role in chaperoning any misfolded proteins involved in regulated exocytosis. The complexity, but yet high fidelity, of presynaptic exocytosis requires that the participating proteins are in an optimal conformation. The vast array of protein–protein interactions involved in exocytosis probably requires the transition of some proteins through several conformations, resulting in a requirement for molecular chaperones to prevent misfolding and promiscuous interactions of such proteins. Therefore, future work will be required to provide further understanding of the protein interactions made by Csp and give a more detailed picture of how Csp acts to chaperone components of the membrane fusion machinery during the process of neurotransmitter release. It is clear, however, that the focus of attention will be on interactions of Csp with the components of the essential SNARE complex and the chaperone effects of Csp on these SNARE proteins.

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