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The cysteine string protein multimeric complex

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Abstract

Cysteine string protein (CSPα) is a member of the cellular folding machinery that is located on regulated secretory vesicles. We have previously shown that CSPα in association with Hsc70 (70 kDa heat shock cognate protein) and SGT (small glutamine-rich tetratricopeptide repeat domain protein) is a guanine nucleotide exchange factor (GEF) for $G_{\alpha s}$. Association of this CSPα complex with N-type calcium channels, a channel key in coupling calcium influx with synaptic vesicle exocytosis, triggers tonic G protein inhibition of the channels. Syntaxin 1A, a plasma membrane SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) critical for neurotransmission, coimmunoprecipitates with the CSPα/G protein/N-type calcium channel complex, however the significance of syntaxin 1A as a component of this complex remains unknown. In this report, we establish that syntaxin 1A interacts with CSPα, Hsc70 as well as the synaptic protein interaction (synprint) region of N-type channels. We demonstrate that huntingtin^{exon1}, a putative biologically active fragment of huntingtin, displaces both syntaxin 1A and CSPα from N-type channels. Identification of the protein components of the CSPα/GEF system is essential in establishing its precise role in synaptic transmission.

Keywords: CSPα; Syntaxin 1A; N-type calcium channel; Hsc70; SGT; G protein

Cysteine string protein (CSPα) is a 34 kDa member of the large, evolutionarily conserved J protein family also called the DnaJ or Hsp40 (heat shock protein 40) protein family (Fig. 1A) [1]. The importance of the J protein family in protein folding has been recognized for many years (reviewed: [2,3]). In humans, over 40 J protein family members, classified as type I, II, and III, have been identified [4,5]. Each member of the J protein family contains a 70 amino acid region of homology comprised of four α helices with a highly conserved tripeptide of histidine, proline, and aspartic acid (HPD motif) located between helices II and III, called a ''J domain''. Outside of the J domain, J proteins are divergent. J proteins act in concert with the Hsp70/Hsc70 (70-kDa heat shock protein and cognate protein) chaperone family to regulate the conformation of substrate proteins. Via their J domain, J proteins recruit and stimulate the ATPase activity of Hsp70s, thereby harnessing the ATPase activity for conformational work on specific proteins (e.g. recycling of clathrin coats by auxilin). Substrates of Hsc70 do not share homology but typically demonstrate significant conformational flexibility. In most cases, the mammalian targets of the J protein/Hsp70 chaperone complexes remain unknown. CSPα, a type III J protein family member, is expressed on synaptic vesicles [6] and clathrin-coated vesicles [7] in neurons as well as exocrine [1,8], endocrine [9], and neuroendocrine [10,11] secretory granules but no obvious CSPα homolog is found in *Saccharomyces cerevisiae* [12]. In addition to the signature 'J domain', CSPα contains a distinctive cysteine string region, a linker region, and a C terminus region that is not conserved throughout evolution. Most of the cysteine residues are palmitoylated and are required for membrane attachment to the secretory vesicle [13,14] and may underlie the association of CSPα with caveolin-containing lipid raft fractions [15]. Via its J domain CSPα interacts with Hsc70 and enhances its ATPase activity [16,17]. Together with Hsc70 and SGT (small glutamine-rich tetratricopeptide repeat domain protein), CSPα assembles into an

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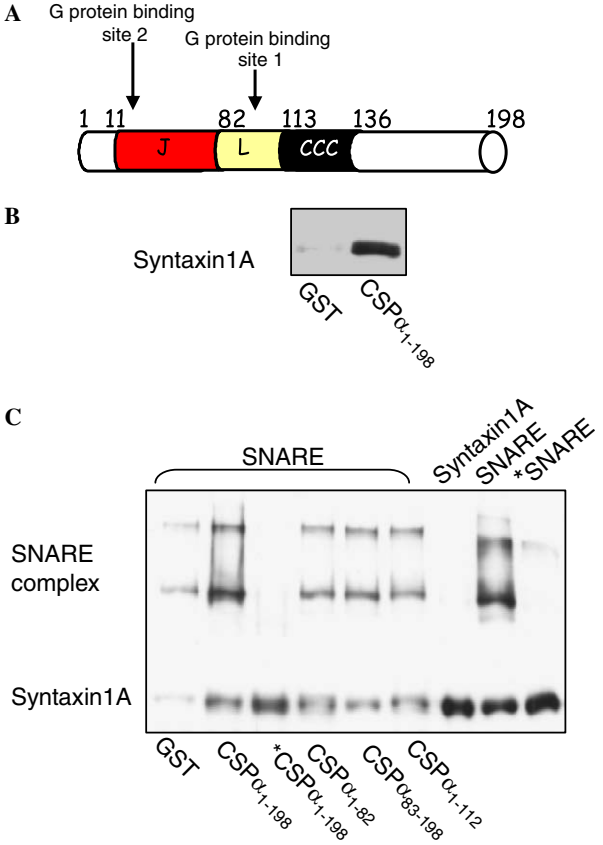

Fig. 1. CSPα interacts with syntaxin 1A and the SNARE complex. (A) A schematic representation of rat CSPα. J, J domain; L, linker region; CCC, cysteine string region. (B) CSPα was immobilized, bound syntaxin 1A was eluted in sample buffer and identified by Western blotting with anti-syntaxin (Sigma). (C) SNARE proteins VAMP2, syntaxin 1A, and SNAP25 were purified and incubated overnight at 4 °C to form SNARE complexes. GST and GST-tagged constructs of full-length CSPα, $CSP\alpha_{1-82}$, $CSP\alpha_{83-198}$, and $CSP\alpha_{1-112}$ were purified and immobilized on glutathione-Sepharose beads. SNARE complexes were incubated with the immobilized GST-tagged proteins at 4 °C. The beads were washed, and bound proteins were eluted in sample buffer, fractionated by SDS–PAGE, and analyzed by Western blotting with an anti-syntaxin antibody (Sigma). In the last three lanes syntaxin 1A, and SNARE complexes were loaded directly on the gel. A star indicates samples that were heated at 80 °C for 15 min (which disrupts SNARE complexes) prior to loading on the gel.

enzymatically active chaperone complex [18,19]. The presence of this chaperone complex on secretory vesicles suggests that CSPα is a coordinating anchor in a regulatory step involving conformation/activity changes in client protein(s) critical in exocytosis.

CSPα is a critical component in synaptic transmission. Deletion of the CSPα gene severely impairs central and presynaptic transmission in *Drosophila melanogaster* [20–22] as does mutagenesis of Hsc70-4 [23]. The *Drosophila* CSPα null mutants die as larvae or within days of adulthood. The small number of flies that survive to adulthood are characterized by uncoordinated sluggish movements, shaking, and temperature-sensitive paralysis [20]. Deletion of CSPα in mice causes no significant change at birth but a progressive neurodegeneration ensues, with no survival beyond four months [24,25]. In the absence of CSPα, it is predicted that Hsc70 will not be localized to synaptic vesicles, thereby rendering Hsc70 folding activity mechanically ineffective at this cellular locale. In the CSPα mutants it appears that the capacity of the protein quality machinery is exceeded and misfolded synaptic proteins accumulate to dangerous levels triggering neurodegeneration, however the precise sequence of pathogenic events remains to be identified. A direct demonstration of the action of misfolded proteins on CSPα activity was shown in human endothelial kidney cells expressing either mutant (misfolded) or native (control) $huntingtin^{exon1}$. In this study, we found that CSPα activity is inhibited when levels of misfolded proteins (i.e. mutant $huntingtin^{exon1}$) are increased [26] providing more conclusive support that CSPα is involved in protein conformation remodeling and that the capacity of the CSPα machinery can be exhausted by increased levels of misfolded proteins. Folding activity of the CSPα complex may be dynamic. Expression of CSPα is reported to be modified by antidepressants [27–31], amphetamines [32], and diabetes [33] while Hsc70 expression is induced by synaptic activation and other types of cell stress (reviewed: [34]). These studies indicate that changes in CSPα chaperone activity due to altered protein levels or activities have significant consequences.

What are the authentic substrate(s) for the CSPα chaperone complex? It is possible that the CSPα chaperone complex is a dedicated complex with one primary target however we cannot rule out the possibility that the CSPα complex regulates the conformation/activity of several client proteins. A number of possible targets have been proposed based on their association with CSPα including; G proteins [19,35], vertebrate voltage-dependent calcium channels [35,36], syntaxin 1A [37,38] however see [36,39], VAMP/synaptobrevin (vesicle associated membrane protein) [36,40], and synaptotagmin I [41]. In addition, CSPα–CSPα interactions have been identified [42]. Furthermore, $G_{\alpha s}$ activity is regulated by the CSPα complex [19]. Exactly what conformational change is achieved and how the conformational changes are transmitted are not yet known for G proteins or other potential substrates. Hsc70s are implicated in diverse transactions with substrate proteins and most of these conformational steps involve Hsp40s other than CSPα (e.g. auxilin, HDJ-1, etc.) In fact, Hsc70 is proposed to play a role in the conformational demands of several trafficking events including: recycling of clathrin coats [43], exocrine granule exocytosis (protection against pancreatitis) [44], ER to Golgi transport [45], *Xenopus* cortical granule exocytosis [46], endosomal trafficking [47], and proteasome trafficking [48], and is also a candidate chaperone in synaptic plasticity. In addition, several studies have implicated Hsc70 in the modulation of protein aggregation (reviewed; [49]). Identification of the precise conformational change(s) achieved by the CSPα/Hsc70/SGT complex remains challenging given Hsc70s' versatility.

We have recently shown that in association with Hsc70 and SGT, CSPα regulates heterotrimeric GTP-binding protein (G protein) function by preferentially targeting the inactive GDP-bound form of $G_{\alpha s}$ and promoting GDP/GTP exchange which increases cAMP [19]. CSPα is selective for $G_{\alpha s}$, and, as such, is the first identified GEF (guanine nucleotide exchange factor) for $G_{\alpha s}$. In addition, CSPα enhances G protein inhibition of N-type calcium channels [35,50], through interactions with the synprint (synaptic protein interaction) region of the N-type calcium channel and G protein βγ subunits. The downstream effects of the CSPα-induced increase in cAMP remain to be established. However, CSPα has been implicated in the modulation of transmembrane calcium flux [51–53] as well as exocytosis [9,54–60] and signaling through $G_{\alpha s}$ may underlie these events. Co-immunoprecipitation experiments suggest that syntaxin 1A is a component of the CSPα/G protein/N-type calcium channel complex [35], suggesting a link between G protein signaling and the exocytotic machinery. Although, the molecular basis of syntaxin 1A's involvement with this complex remains unresolved. In this study, we address the association of syntaxin 1A with the complex. We demonstrate that syntaxin 1A binds to CSPα and Hsc70 as well as the synprint region of N-type calcium channels. These studies indicate that the CSPα chaperone/GEF system operates in conjunction with syntaxin 1A.

Materials and methods

Preparation of fusion proteins. The glutathione-*S*-transferase (GST) fusion proteins, cytosolic syntaxin 1A/GST, SNAP-25/GST, VAMP2/GST, Hsc70/GST, SGT/GST, and CSPα/GST, the CSPα truncation mutants, and the His_6 fusion proteins, $Ca_v2.2_{718–963}$ (synprint region) and $Ca_v2.2$ truncation mutants, were prepared as described previously [1,19,61,62]. The Myc-tagged huntingtin with 20 polyglutamines (pGEX-htt_20Q) was prepared as previously described in detail [26]. Recombinant GST fusion proteins were purified from glutathione–Sepharose beads by cleavage with 0.2 μM thrombin in 50 mM Tris, pH 8, 150 mM NaCl, 2.5 mM $CaCl_2$ followed by incubation in 0.3 mM PMSF. His_6 fusion proteins were purified from Ni^{2+} beads with 500 mM imidazole. The concentrations of recombinant proteins were estimated by Coomassie blue staining of SDS–polyacrylamide gels using bovine serum albumin (BSA) as a standard.

In vitro-binding assays. Equal concentrations of the soluble proteins were incubated with equal concentrations of bead-immobilized GST-tagged proteins or control GST for 30 min at 37 °C. For experiments involving SNARE complexes, GST-tagged proteins incorporating the full cytoplasmic domains of VAMP2, SNAP25, and syntaxin 1A were purified and their GST tags were removed by thrombin cleavage. The SNARE protein concentrations were then determined by SDS–PAGE using BSA as a standard. Two micromolars of each SNARE proteins was incubated together at 4 °C overnight. Preformed SNARE complexes were then incubated with equal concentrations of immobilized CSPα/GST and CSPα/GST truncation mutants, SGT/GST and Hsc70/GST for 30 min at 4 °C. The beads were washed and bound proteins were eluted in sample buffer, fractionated by SDS–PAGE, and analyzed by Western blotting.

Immunoblotting. Proteins were transferred electrophoretically at constant voltage from polyacrylamide gels to nitrocellulose (0.45 μm) in 20 mM Tris, 150 mM glycine, and 12% methanol. Transferred proteins were visualized by Ponceau S. Nitrocellulose membranes were blocked for non-specific binding using a 5% milk, 0.1% Tween 20, PBS solution and incubated with primary antibody overnight at 4 °C or for 2 h at room temperature. The membranes were washed in the blocking solution and incubated with horseradish peroxidase-coupled secondary antibody. Antigen was detected using chemiluminescent horseradish peroxidase substrate (ECL, Amersham Biosciences), followed by exposure to Amersham Biosciences Hyperfilm-MP. For competition experiments, bound anti-sera were quantified by BioRad Fluor-S MultiImager Max and Quantity One 4.2.1 software and values were expressed as mean ± standard error of at least three independent experiments.

Results

CSPα binds both syntaxin 1A and the SNARE complex

Protein–protein interactions between CSP and syntaxin have been reported [37,38,63,64] although this finding has been controversial [36,39]. We have previously shown that anti-syntaxin monoclonal antibody immunoprecipitates the CSPα complex from rat brain homogenates [35]. To investigate the possible direct association between the CSPα complex and syntaxin 1A, glutathione-*S*-transferase (GST) fusion proteins consisting of full-length CSPα coupled to glutathione beads were used in an in vitro-binding assay. The beads were incubated with equal amounts of purified soluble syntaxin 1A. CSPα was found to directly and specifically interact with syntaxin 1A (Fig. 1B).

Syntaxin 1A assembles with VAMP2/synaptobrevin (vesicle associated membrane protein) and SNAP25 (synaptosomal associated protein of 25 kDa) into a four helical bundle structure known as the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complex. The SNARE complex is critical for neurotransmitter release. Resistance to disruption by SDS is a hallmark feature of the SNARE complex and is thought to be representative of the extreme stability of this complex in intact neurons. The SDS-resistance of the SNAP25/VAMP2/syntaxin complex results in a shift of the apparent molecular masses of syntaxin1A, SNAP25, and VAMP2 upon SDS–PAGE from monomers to higher molecular weight multimers if the samples are not boiled prior to electrophoresis. To determine whether CSPα interacts with SNARE complexes in addition to syntaxin 1A, we performed in vitro-binding assays between SNARE complexes and immobilized CSPα constructs (Fig. 1C). Briefly, the SNARE proteins VAMP2, syntaxin 1A, and SNAP25 were purified and incubated overnight to form SNARE complexes, which were then incubated with bead-immobilized GST and GST-tagged full-length CSPα, $CSP\alpha_{1–82}$, $CSP\alpha_{83–198}$, and $CSP\alpha_{1–112}$. The beads were washed, and bound proteins were eluted in sample buffer, fractionated by SDS–PAGE, and analyzed by Western blotting with an anti-syntaxin antibody. In agreement with previous studies [65], SNAREs formed SDS resistant complexes that were disrupted upon incubation at 80 °C for 15 min. Fig. 1C demonstrates that the SNARE complex binds to $CSP\alpha_{1–198}$ and weaker binding was observed with CSPα truncation mutants $CSP\alpha_{1–82}$, $CSP\alpha_{83–198}$, and $CSP\alpha_{1–112}$.

CSPα and syntaxin 1A bind two regions of N-type calcium channels

N-type channels are composed of a pore-forming $Ca_v2.2$ subunit with β and α_2-δ modulatory subunits. The $Ca_v2.2$ subunit is shown schematically in Fig. 2A. Syntaxin 1A [66,67] and CSPα [35] both bind N-type calcium channels at the synprint region, located within the domain II-III intracellular linker of the $Ca_v2.2$ subunit, and enhance G protein regulation of N-type channels [35]. Syntaxin 1A interacts with synprint at two sites [62,66,68]. To begin to understand the structural requirements for CSPα's interaction with synprint, four synprint truncation mutants were constructed, expressed, and purified (Fig. 2B). Equal concentrations of the soluble $His_6Ca_v2.2$ truncation mutants were incubated with the immobilized CSPα/GST in an in vitro-binding assay. Bound proteins were eluted in sample buffer, fractionated by SDS–PAGE, and analyzed by Western blotting using anti-Xpress monoclonal antibody. Fig. 2C demonstrates that the binding to full-length synprint and $His_6Ca_v2.2_{718–919}$ is robust. Binding to $His_6Ca_v2.2_{718–869}$ is substantially reduced, whereas deletion of a further 50 amino acids ($His_6Ca_v2.2_{718–820}$) completely abolishes binding. We also observed binding of $His_6Ca_v2.2_{821–963}$ to CSPα (Fig. 2C). These results indicate that CSPα interacts with two sites within the synprint region. Our study suggests that the regions of synprint to which CSPα binds overlap or are very closely adjacent to the regions previously shown to bind syntaxin 1A [62,66,69].

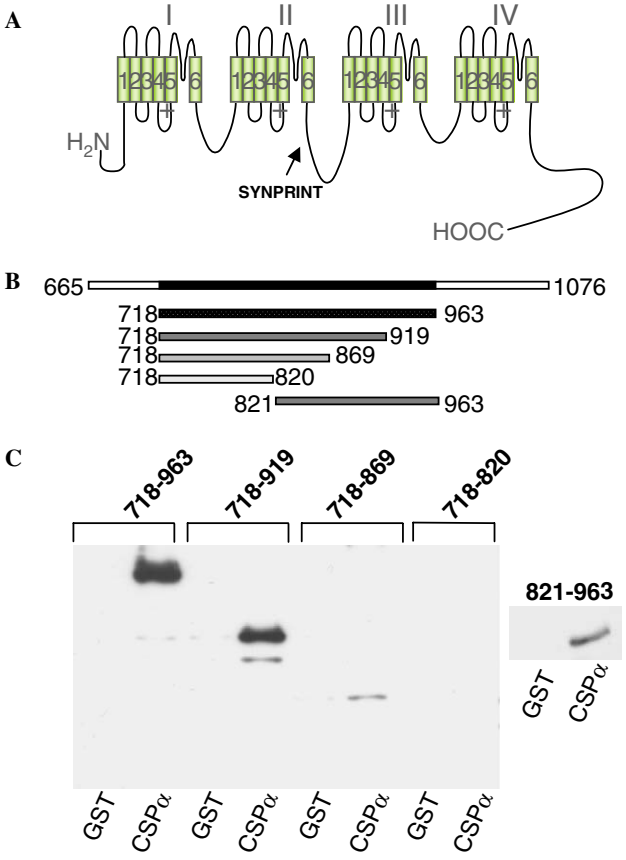

Fig. 2. Binding of $His_6Ca_v2.2$ synprint truncation mutants to CSPα/GST. (A) Membrane topology of the $Ca_v2.2$ subunit of the N-type calcium channel. (B) Diagram of $Ca_v2.2$ synprint truncation mutants. (C) Imidazole-eluted full-length $His_6Ca_v2.2$ synprint and $His_6Ca_v2.2$ synprint truncation mutants ($His_6Ca_v2.2_{718–919}$, $His_6Ca_v2.2_{718–869}$, $His_6Ca_v2.2_{718–820}$, and $His_6Ca_v2.2_{821–963}$) were incubated with CSPα-GST or GST immobilized on glutathione-Sepharose beads for 30 min at 37 °C. Results were determined by Western blotting with anti-Xpress monoclonal antibody (Invitrogen) which recognizes a sequence expressed in the N-terminal His_6 tag region (Thr-Leu-Tyr-Asp-Asp-Asp-Asp-Lys). Note that the visualization of $His_6Ca_v2.2_{821–963}$ (right panel) required longer exposure times.

Direct evaluation of syntaxin 1A and CSPα competition for synprint is confounded by the capacity of syntaxin 1A and CSPα to bind to one another (Figs. 1 and 3A). However, we compared their synprint binding using huntingtin^{exon1}, another synprint-binding protein that does not interact with either CSPα or syntaxin 1A [62]. Huntingtin is a cytosolic protein targeted by specific proteases. Proteolytic huntingtin fragments are thought to have their own discreet physiological functions and N-terminal huntingtin fragments (i.e. huntingtin^{exon1}) have been shown to compete with syntaxin 1A for association with synprint [62]. Although huntingtin^{exon1} like syntaxin 1A interacts with synprint, it does not trigger G protein inhibition of the channel. Increasing concentrations of thrombin cleaved soluble CSPα were preincubated with equal amounts of solubilized synprint, followed by the addition of immobilized huntingtin^{exon1}, the beads were washed, bound proteins were eluted, and $Ca_v2.2$ synprint binding was assessed by Western blot. Fig. 3B shows that CSPα (like syntaxin 1A) blocks the association of huntingtin^{exon1} with synprint in a concentration-dependent manner. In Fig. 3C, for comparison purposes, CSPα (from Fig. 3B) and syntaxin 1A (from [62]) competition of the huntingtin^{exon1}/synprint association is shown in graphical form. Our results indicate that CSPα, syntaxin 1A, and huntingtin^{exon1} associate with the same region of synprint; syntaxin 1A has a greater affinity for synprint than CSPα.

Next we evaluated the effect of the other CSPα-binding proteins Hsp70 and SGT on the CSPα/synprint interaction. CSPα-GST and GST were immobilized on glutathione-Sepharose, and preincubated with Hsp70, SGT, and ATP, as indicated in Fig. 3D, before the addition of equal amounts of solubilized synprint. The beads were washed, bound proteins were eluted, and $Ca_v2.2$ synprint binding to CSPα-GST was assessed by Western blot. Neither Hsc70 nor SGT influenced the interaction of CSPα with synprint (Fig. 3D). The trimeric chaperone complex CSPα/Hsc70/SGT previously shown to modulate G protein function by promoting GDP/GTP exchange did not alter CSPα/synprint association.

Finally we examined a possible Hsc70/syntaxin 1A association. GST fusion proteins of CSPα, Hsc70, and SGT were incubated with equal amount of soluble syntaxin 1A. Syntaxin 1A was found to interact with Hsc70 in addi-

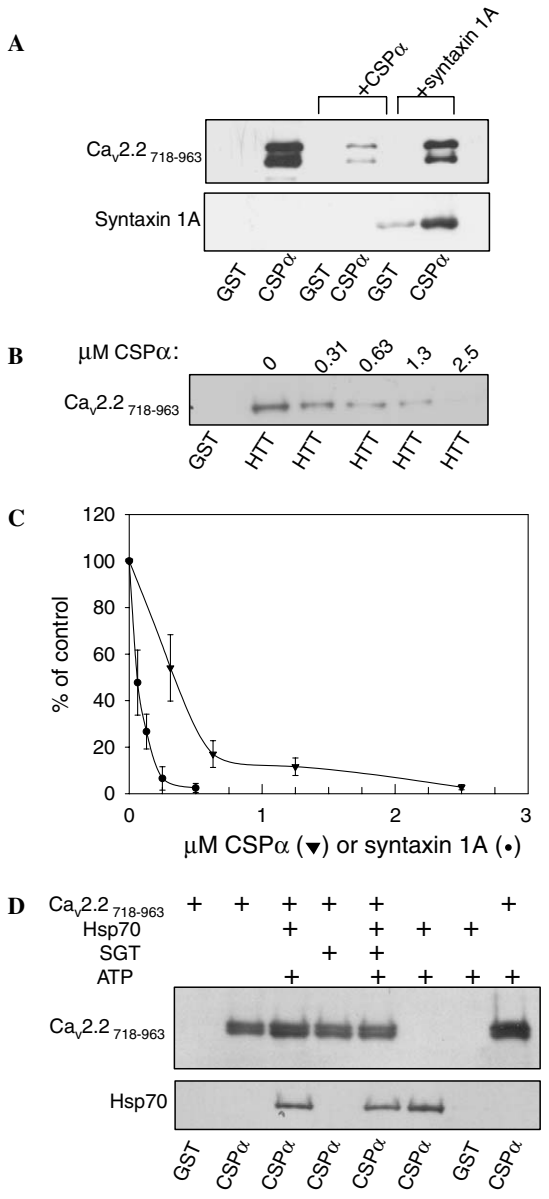

Fig. 3. The interaction of CSPα and syntaxin 1A with synprint. (A) Immunoblot analysis showing binding of soluble $His_6Ca_v2.2_{665–1076}$ to immobilized CSPα-GST and GST in the presence and absence of soluble CSPα or syntaxin 1A. Results were determined by Western blotting with anti-Xpress monoclonal antibody. (B) Increasing concentrations of thrombin cleaved soluble CSPα were preincubated with soluble $His_6Ca_v2.2$ synprint for 15 min at 37 °C. The pre-incubation mixtures were then added to immobilized $huntingtin^{exon1(20Q)}$ and these were incubated together for 30 min at 37 °C. The beads were washed and bound proteins were eluted in sample buffer, fractionated using SDS–PAGE, and analyzed by Western blotting using anti-Xpress monoclonal antibody (Invitrogen). (C) Concentration–response curve from the data in part (B) and for syntaxin 1A (Ref. [62], with permission from Elsevier.) (D) Immunoblot analysis showing binding of soluble $His_6Ca_v2.2$ synprint to immobilized CSPα-GST or GST. Immobilized proteins were preincubated with Hsp70, SGT, and 2 mM ATP as indicated for 30 min before incubation with synprint for an additional 30 min at 37 °C. The beads were washed and bound proteins were eluted in sample buffer, fractionated using SDS–PAGE, and analyzed by Western blotting using anti-Xpress monoclonal (Invitrogen) and anti-Hsp70 (Sigma).

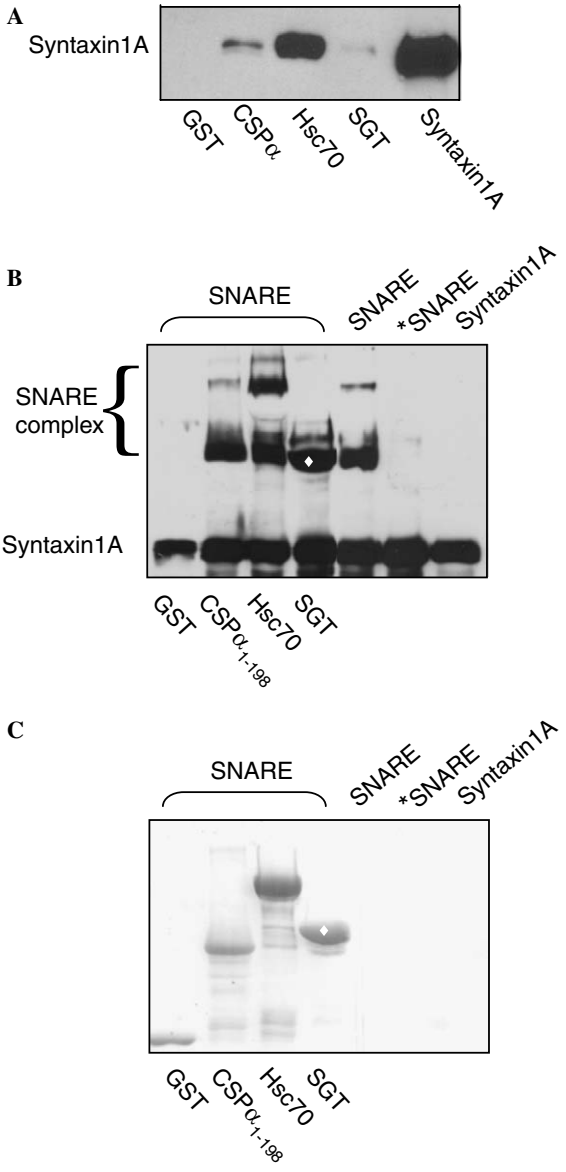

Fig. 4. Hsc70 interacts with syntaxin 1A and the SNARE complex. (A) Immunoblot analysis showing binding of soluble syntaxin 1A to immobilized GST, CSPα-GST, Hsc70-GST, SGT-GST. Results were determined by Western blotting with anti-syntaxin monoclonal antibody (Sigma). (B) SNARE complexes were formed overnight at 4 °C, incubated with immobilized GST-tagged constructs of CSPα, Hsc70, and SGT, and associated proteins identified by Western blotting with anti-syntaxin antibody (Sigma). In the last three lanes syntaxin 1A, and SNARE complexes were loaded directly on the gel. A star indicates samples that were heated at 80 °C for 15 min prior to loading on the gel. (C) Ponceau S profile is shown. ◊ indicates recombinant GST–SGT that is non-specifically detected by anti-syntaxin.

tion to CSPα. The Hsc70/syntaxin 1A interaction was robust and specific (Fig. 4A). Furthermore, Hsc70 was found to bind to the SNARE complex in addition to monomeric syntaxin 1A. GST-Hsc70 increased the assembly of syntaxin 1A into the SNARE complex (Fig. 4B). The association between SGT and syntaxin 1A was observed to be very weak. The band labeled with ◊ represents cross-reactivity of anti-syntaxin monoclonal with the

abundant recombinant protein GST–SGT rather than syntaxin 1A (see Ponceau S for comparison) (Fig. 4C). Taken together, these results demonstrate that syntaxin 1A is a component of the CSPα chaperone system.

Discussion

J protein and Hsp70 families are implicated in diverse cellular functions, however the common mechanism underlying these functions is thought to be the ability to bind hydrophobic polypeptide stretches of client proteins and elicit conformational changes. The identification of specific J protein/Hsp70 chaperone pairs and the events that they regulate *in vivo* remains a central biological question. One of the important neural J protein/Hsp70 chaperone machines is the CSPα/SGT/Hsc70 complex, a complex that is critical in neurotransmission [20,25]. While the basic mechanism of the CSPα/SGT/Hsc70 complex is most likely universal, we expect the non-conserved regions of CSPα to confer specialization. The precise role of this complex is unknown. In particular, it has been unclear if syntaxin 1A is a component of the CSPα complex. In this study, we provide evidence that CSPα directly associates with syntaxin 1A (Fig. 1A), the SNARE complex (Fig. 1B), and two regions of synprint (Figs. 2 and 3), a key regulatory site of vertebrate N-type calcium channels. We show that syntaxin 1A and CSPα bind to similar regions of synprint and that the synprint-binding protein huntingtin^{exon1} competes with both syntaxin 1A and CSPα for synprint binding (Fig. 3). Assembly of the active trimeric CSPα/Hsc70/SGT chaperone complex does not alter the CSPα/synprint association (Fig. 3), suggesting that the active complex is anchored to the synprint site of N-type calcium channels. Hsc70 is known to manifest its chaperone activity in several different contexts, by transiently binding unrelated protein targets and preventing their unproductive aggregation. Here we report that Hsc70 also associates with syntaxin 1A and the SNARE complex further implicating syntaxin 1A as either (1) a target of chaperone folding activity or (2) a downstream target of the $G_{\alpha s}$ signaling cascade. Consistent with our findings, CSPα knockout mice have been shown to have reduced SNARE complex assembly [24].

Synaptic transmission is under the control of several intracellular messenger pathways. One such pathway, which is important in the modulation of synaptic transmission, is the regulation of N-type calcium channels by heterotrimeric GTP-binding proteins (G proteins). G-protein-coupled receptors respond to extracellular stimuli by activating G proteins. The activation involves an exchange of GDP for GTP on G_{α} subunits and release of G_{α}GTP and $G_{\beta\gamma}$ to interact with their effectors. $G_{\beta\gamma}$ inhibits N-type calcium channels, a channel critical in coupling calcium influx with synaptic vesicle exocytosis. G proteins are themselves under the control of yet other regulators such as RGS (regulator of G protein signaling) and GEF proteins. The CSPα/Hsc70/SGT chaperone complex has emerged as a GEF [19,35]. CSPα has a number of functional parallels with a structurally distinct protein called Ric-8A (synembryn). CSPα and Ric-8A are both abundant neural GEFs that are implicated in neuronal transmitter release [1,70]. Deletion of either CSPα or Ric-8 causes paralysis. CSPα is a GEF for $G_{\alpha s}$ but not $G_{\alpha i1}$, while Ric-8A is a GEF for $G_{\alpha i1}$, $G_{\alpha q}$, and $G_{\alpha o}$ but not $G_{\alpha s}$. Thus, it seems that CSPα, like Ric-8, is responsible for integrating synaptic G protein signaling pathways. Our results suggest that association of CSPα with synprint targets the CSPα chaperone system to G proteins located near N-type channels.

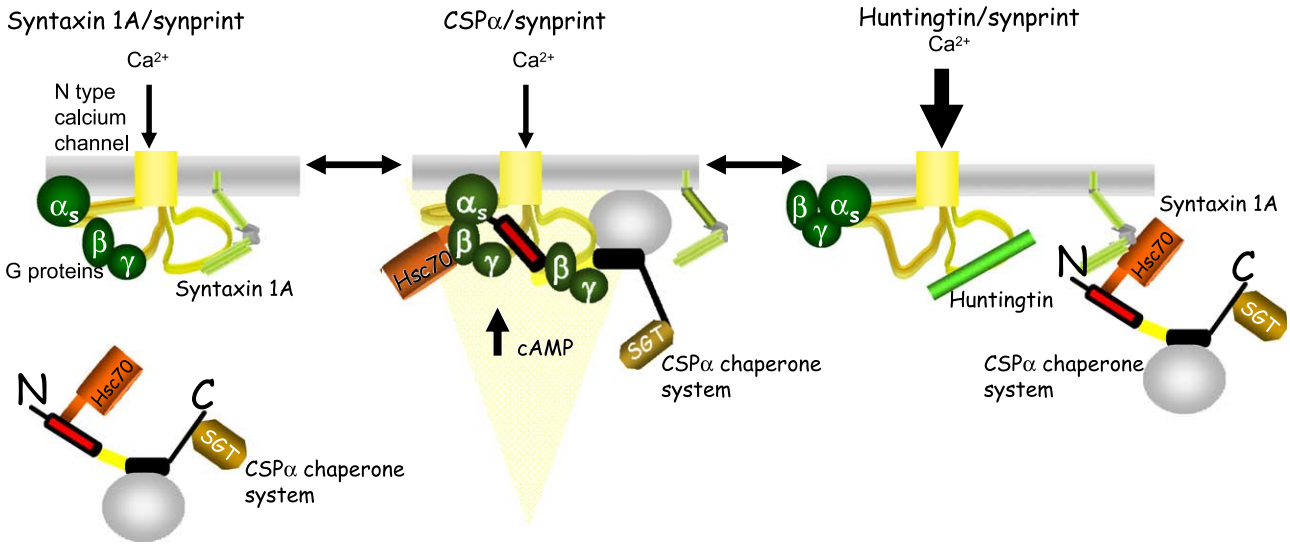

Fig. 5. Current working model of CSPα interactions. The synaptic vesicle protein, CSPα, when activated by Hsc70 and SGT, promotes guanine nucleotide exchange of $G_{\alpha s}$. The active GEF/chaperone complex associates with the synprint region of the N-type calcium channel, located within the domain II–III linker, to trigger the $G_{\alpha s}$-mediated increase in cellular [cAMP] and the $G_{\beta\gamma}$-mediated inhibition of the channel. Syntaxin 1A associates with CSPα, Hsc70 and synprint. Syntaxin 1A and huntingtin N-terminal fragments compete with CSPα for association with N-type channels. Huntingtin N-terminal fragments do not modulate G protein signaling.

A current working model demonstrating the known interactions of the CSPα multimeric complex is illustrated in Fig. 5. In this model, CSPα, when in close proximity to the active zone, binds G proteins and N-type calcium channels. In the presence of Hsc70 and SGT, CSPα is activated and promotes GDP/GTP exchange of $G_{\alpha s}$, increases [cAMP], and enhances $G_{\beta\gamma}$ inhibition of N-type channels [19]. The GEF activity of CSPα involves two G protein-binding sites. Site 1 (residues 83–113) binds G_{α} and $G_{\beta\gamma}$, while site 2 (residues 1–82) binds G_{α}. Both huntingtin^{exon1} and syntaxin 1A compete with the CSPα system for association with N-type calcium channels. Our model proposes that syntaxin 1A is a dynamic component of the CSPα complex interacting with CSPα, Hsc70 as well as N-type calcium channels. Further investigation is required to establish the precise events underlying CSPα modulation of $G_{\alpha s}$ signaling and the role of syntaxin 1A in this process.

In most cases the role of mammalian J domain/Hsp70 chaperone complexes remains unknown. A major endeavor of current inquiry is to understand the biochemical events underlying the function of individual J proteins. The identification of the CSPα/syntaxin 1A, CSPα/synprint, Hsc70/syntaxin 1A, syntaxin 1A/synprint protein–protein interactions is an important step in that direction. It is now possible to further investigate the mechanisms whereby the CSPα chaperone machinery participates in neurotransmitter release.

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References

[1] J.E. Braun, R.H. Scheller, Cysteine string protein, a DnaJ family member, is present on diverse secretory vesicles, Neuropharmacology 34 (1995) 1361–1369.
[2] J.C. Young, V.R. Agashe, K. Siegers, F.U. Hartl, Pathways of chaperone-mediated protein folding in the cytosol, Nat. Rev. Mol. Cell Biol. 5 (2004) 781–791.
[3] P. Walsh, D. Bursac, Y.C. Law, D. Cyr, T. Lithgow, The J-protein family: modulating protein assembly, disassembly and translocation, EMBO Rep. 5 (2004) 567–571.
[4] C.Y. Fan, S. Lee, D.M. Cyr, Mechanisms for regulation of Hsp70 function by Hsp40, Cell Stress Chaperones. 8 (2003) 309–316.
[5] M.E. Cheetham, A.J. Caplan, Structure, function and evolution of DnaJ: conservation and adaptation of chaperone function, Cell Stress Chaperones 3 (1998) 28–36.
[6] A. Mastrogiacomo, S.M. Parsons, G.A. Zampighi, D.J. Jenden, J.A. Umbach, C.B. Gundersen, Cysteine string proteins: a potential link between synaptic vesicles and presynaptic Ca2+ channels, Science 263 (1994) 981–982.
[7] F. Blondeau, B. Ritter, P.D. Allaire, S. Wasiak, M. Girard, N.K. Hussain, A. Angers, V. Legendre-Guillemin, L. Roy, D. Boismenu, R.E. Kearney, A.W. Bell, J.J. Bergeron, P.S. McPherson, Tandem MS analysis of brain clathrin-coated vesicles reveals their critical involvement in synaptic vesicle recycling, Proc. Natl. Acad. Sci. USA 101 (2004) 3833–3838.
[8] C.M. Zhao, G. Jacobsson, D. Chen, R. Hakanson, B. Meister, Exocytotic proteins in enterochromaffin-like (ECL) cells of the rat stomach, Cell Tissue Res. 290 (1997) 539–551.
[9] H. Brown, O. Larsson, R. Branstrom, S. Yang, B. Leibiger, I. Leibiger, G. Fried, T. Moede, J.T. Deeney, G.R. Brown, G. Jacobsson, C.J. Rhodes, J.E. Braun, R.H. Scheller, B.E. Corkey, P. Berggren, B. Meister, Cysteine string protein (CSP) is an insulin secretory granule-associated protein regulating beta-cell exocytosis, EMBO J. 17 (1998) 5048–5058.
[10] S.A. Kohan, M. Pescatori, N.C. Brecha, A. Mastrogiacomo, J.A. Umbach, C.B. Gundersen, Cysteine string protein immunoreactivity in the nervous system and adrenal gland of rat, J. Neurosci. 15 (1995) 6230–6238.
[11] L.H. Chamberlain, J. Henry, R.D. Burgoyne, Cysteine string proteins are associated with chromaffin granules, J. Biol. Chem. 271 (1996) 19514–19517.
[12] L.H. Chamberlain, R.D. Burgoyne, Cysteine string protein: the chaperone at the synapse, J. Neurochem. 74 (2000) 1781–1789.
[13] C.B. Gundersen, A. Mastrogiacomo, K. Faull, J.A. Umbach, Extensive lipidation of a Torpedo cysteine string protein, J. Biol. Chem. 269 (1994) 19197–19199.
[14] L.H. Chamberlain, R.D. Burgoyne, The cysteine-string domain of the secretory vesicle cysteine-string protein is required for membrane targeting, Biochem. J. 335 (Pt. 2) (1998) 205–209.
[15] J.M. Magga, J.G. Kay, A. Davy, N.P. Poulton, S.M. Robbins, J.E.A. Braun, ATP dependence of the SNARE/caveolin1 interaction in the hippocampus, Biochem. Biophys. Res. Commun. 291 (2002) 1232–1238.
[16] J.E. Braun, S.M. Wilbanks, R.H. Scheller, The cysteine string secretory vesicle protein activates Hsc70 ATPase, J. Biol. Chem. 271 (1996) 25989–25993.
[17] L.H. Chamberlain, R.D. Burgoyne, Activation of the ATPase activity of heat-shock proteins Hsc70/Hsp70 by cysteine-string protein, Biochem. J. 322 (Pt. 3) (1997) 853–858.
[18] S. Tobaben, P. Thakur, R. Fernandez-Chacon, T.C. Sudhof, J. Rettig, B. Stahl, A trimeric protein complex functions as a synaptic chaperone machine, Neuron 31 (2001) 987–999.
[19] M. Natochin, T.N. Campbell, B. Barren, L.C. Miller, S. Hameed, N.O. Artemyev, J.E. Braun, Characterization of the G alpha s regulator cysteine string protein, J. Biol. Chem. 280 (2005) 30236–30241.
[20] K.E. Zinsmaier, K.K. Eberle, E. Buchner, N. Walter, S. Benzer, Paralysis and early death in cysteine string protein mutants of *Drosophila*, Science 263 (1994) 977–980.
[21] J.A. Umbach, K.E. Zinsmaier, K.K. Eberle, E. Buchner, S. Benzer, C.B. Gundersen, Presynaptic dysfunction in *Drosophila* csp mutants, Neuron 13 (1994) 899–907.
[22] J.W. Barclay, H.L. Atwood, R.M. Robertson, Impairment of central pattern generation in *Drosophila* cysteine string protein mutants, J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. 188 (2002) 71–78.
[23] P. Bronk, J.J. Wenniger, K. Dawson-Scully, X. Guo, S. Hong, H.L. Atwood, K.E. Zinsmaier, Drosophila Hsc70-4 is critical for neurotransmitter exocytosis in vivo, Neuron 30 (2001) 475–488.
[24] S. Chandra, G. Gallardo, R. Fernandez-Chacon, O.M. Schluter, T.C. Sudhof, alpha-Synuclein cooperates with CSPalpha in preventing neurodegeneration, Cell 123 (2005) 383–396.
[25] R. Fernandez-Chacon, M. Wolfel, H. Nishimune, L. Tabares, F. Schmitz, M. Castellano-Munoz, C. Rosenmund, M.L. Montesinos, J.R. Sanes, R. Schneggenburger, T.C. Sudhof, The synaptic vesicle protein CSP alpha prevents presynaptic degeneration, Neuron 42 (2004) 237–251.

[26] L.C. Miller, L.A. Swayne, L. Chen, Z.P. Feng, J.L. Wacker, P.J. Muchowski, G.W. Zamponi, J.E.A. Braun, Cysteine string protein (CSP) inhibition of N-type calcium channels is blocked by mutant huntingtin, J. Biol. Chem. 278 (2003) 53072–53081.
[27] M. Yamada, S. Yamazaki, K. Takakashi, K. Nara, H. Ozawa, S. Yamada, Y. Kiuchi, K. Oguchi, K. Kamijima, T. Higuchi, K. Momose, Induction of cysteine string protein after chronic antidepressant treatment in rat frontal cortex, Neurosci. Lett. 301 (2001) 183–186.
[28] M.L. Cordeiro, J.A. Umbach, C.B. Gundersen, Lithium ions upregulate mRNAs encoding dense-core vesicle proteins in nerve growth factor-differentiated PC12 cells, J. Neurochem. 6 (2000) 2622–2625.
[29] M.L. Cordeiro, C.B. Gundersen, J.A. Umbach, Convergent effects of lithium and valproate on the expression of proteins associated with large dense core vesicles in NGF-differentiated PC12 cells, Neuropsychopharmacology 29 (2004) 39–44.
[30] M.L. Cordeiro, C.B. Gundersen, J.A. Umbach, Dietary lithium induces regional increases of mRNA encoding cysteine string protein in rat brain, J. Neurosci. Res. 73 (2003) 865–869.
[31] M.L. Cordeiro, J.A. Umbach, C.B. Gundersen, Lithium ions enhance cysteine string protein gene expression in vivo and in vitro, J. Neurochem. 74 (2000) 2365–2372.
[32] J.F. Bowyer, D.L. Davies, Changes in mRNA levels for heat-shock/stress proteins (Hsp) and a secretory vesicle associated cysteine-string protein (Csp1) after amphetamine (AMPH) exposure, Ann. N. Y. Acad. Sci. 890 (1999) 314–329.
[33] W. Zhang, A. Khan, C.G. Ostenson, P.O. Berggren, S. Efendic, B. Meister, Down-regulated expression of exocytotic proteins in pancreatic islets of diabetic GK rats, Biochem. Biophys. Res. Commun. 291 (2002) 1038–1044.
[34] K. Ohtsuka, T. Suzuki, Roles of molecular chaperones in the nervous system, Brain Res. Bull. 53 (2000) 141–146.
[35] J.M. Magga, S.E. Jarvis, M.I. Arnot, G.W. Zamponi, J.E. Braun, Cysteine string protein regulates G-protein modulation of N-type calcium channels, Neuron 28 (2000) 195–204.
[36] C. Leveque, S. Pupier, B. Marqueze, L. Geslin, M. Kataoka, M. Takahashi, M. De Waard, M. Seagar, Interaction of cysteine string proteins with the alpha1A subunit of the P/Q-type calcium channel, J. Biol. Chem. 273 (1998) 13488–13492.
[37] Z. Nie, R. Ranjan, J.J. Wenniger, S.N. Hong, P. Bronk, K.E. Zinsmaier, Overexpression of cysteine-string proteins in *Drosophila* reveals interactions with syntaxin, J. Neurosci. 19 (1999) 10270–10279.
[38] M.N. Wu, T. Fergestad, T.e. Lloyd, Y. He, K. Broadie, H.J. Bellen, Syntaxin 1A interacts with multiple exocytic proteins to regulate neurotransmitter release in vivo, Neuron 23 (1999) 593–605.
[39] M.F. Matos, J. Rizo, T.C. Sudhof, The relation of protein binding to function: what is the significance of munc18 and synaptotagmin binding to syntaxin 1, and where are the corresponding binding sites? Eur. J. Cell Biol. 79 (2000) 377–382.
[40] F. Boal, H. Zhang, C. Tessier, P. Scotti, J. Lang, The variable C-terminus of cysteine string proteins modulates exocytosis and protein–protein interactions, Biochemistry 43 (2004) 16212–16223.
[41] G.J.O. Evans, A. Morgan, Phosphorylation-dependent interaction of the synaptic vesicle proteins cysteine string protein and synaptotagmin I, Biochem. J. 364 (2002) 343–347.
[42] L.A. Swayne, C. Blattler, J.G. Kay, J.E.A. Braun, Oligomerization characteristics of cysteine string protein, Biochem. Biophys. Res. Commun. 300 (2003) 921–926.
[43] E. Ungewickell, H. Ungewickell, S.E.H. Holstein, R. Linder, K. Prasad, W. Barouch, B. Martin, L.E. Greene, E. Eisenberg, Role of auxilin in uncoating clathrin-coated vesicles, Nature 378 (1995) 632–635.
[44] A.C. Wagner, H. Weber, L. Jonas, H. Nizze, M. Strowski, F. Fiedler, H. Printz, H. Steffen, B. Goke, Hyperthermia induces heat shock protein expression and protection against cerulein-induced pancreatitis in rats, Gastroenterology 111 (1996) 1333–1342.
[45] A.P. Joglekar, J.C. Hay, Evidence for regulation of ER/Golgi SNARE complex formation by hsc70 chaperones, Eur. J. Cell Biol. 84 (2005) 529–542.
[46] G.B. Smith, J.A. Umbach, A. Hirano, C.B. Gundersen, Interaction between constitutively expressed heat shock protein, Hsc 70, and cysteine string protein is important for cortical granule exocytosis in xenopus oocytes, J. Biol. Chem. 280 (2005) 32669–32675.
[47] M. Girard, V. Poupon, F. Blondeau, P.S. McPherson, The DnaJ-domain protein RME-8 functions in endosomal trafficking, J. Biol. Chem. 280 (2005) 40135–40143.
[48] B. Westhoff, J.P. Chapple, S.J. van der, J. Hohfeld, M.E. Cheetham, HSJ1 is a neuronal shuttling factor for the sorting of chaperone clients to the proteasome, Curr. Biol. 15 (2005) 1058–1064.
[49] P.J. Muchowski, Protein misfolding, amyloid formation, and neurodegeneration: a critical role for molecular chaperones, Neuron 35 (2002) 9–12.
[50] L.C. Miller, L.A. Swayne, J.G. Kay, Z.P. Feng, S.E. Jarvis, G.W. Zamponi, J.E.A. Braun, Molecular determinants of cysteine string protein modulation of N-type calcium channels, J. Cell Sci. 116 (2003) 2967–2974.
[51] C.B. Gundersen, J.A. Umbach, Suppression cloning of the cDNA for a candidate subunit of a presynaptic calcium channel, Neuron 9 (1992) 527–537.
[52] J.A. Umbach, M. Saitoe, Y. Kidokoro, C.B. Gundersen, Attenuated influx of calcium ions at nerve endings of csp and shibire mutant *Drosophila*, J. Neurosci. 18 (1998) 3233–3240.
[53] S. Chen, X. Zheng, K.L. Schulze, T. Morris, H. Bellen, E.F. Stanley, Enhancement of presynaptic calcium current by cysteine string protein, J. Physiol. 538 (2002) 383–389.
[54] L.H. Chamberlain, R.D. Burgoyne, Cysteine string protein functions directly in regulated exocytosis, Mol. Cell Biol. 9 (1998) 2259–2267.
[55] H. Zhang, W.L. Kelley, L.H. Chamberlain, R.D. Burgoyne, J. Lang, Mutational analysis of cysteine-string protein function in insulin exocytosis, J. Cell Sci. 112 (Pt. 9) (1999) 1345–1351.
[56] H. Zhang, W.L. Kelley, L.H. Chamberlain, R.D. Burgoyne, C.B. Wollheim, J. Lang, Cysteine-string proteins regulate exocytosis of insulin independent from transmembrane ion fluxes, FEBS Lett. 437 (1998) 267–272.
[57] M.E. Graham, R.D. Burgoyne, Comparison of cysteine string protein (CSP) and mutant alpha-SNAP overexpression reveals a role for CSP in late steps of membrane fusion in dense-core granule exocytosis in adrenal chromaffin cells, J. Neurosci. 20 (2000) 1281–1289.
[58] M. Morales, A. Ferrus, M. Martinez-Padron, Presynaptic calcium-channel currents in normal and csp mutant *Drosophila* peptidergic terminals, Eur. J. Neurosci. 11 (1999) 1818–1826.
[59] R. Ranjan, P. Bronk, K.E. Zinsmaier, Cysteine string protein is required for calcium secretion coupling of evoked neurotransmission in *Drosophila* but not for vesicle recycling, J. Neurosci. 18 (1998) 956–964.
[60] K. Dawson-Scully, P. Bronk, H.L. Atwood, K.E. Zinsmaier, Cysteine-string protein increases the calcium sensitivity of neurotransmitter exocytosis in *Drosophila*, J. Neurosci. 20 (2000) 6039–6047.
[61] J. Pevsner, S.C. Hsu, J.E. Braun, N. Calakos, A.E. Ting, M.K. Bennett, R.H. Scheller, Specificity and regulation of a synaptic vesicle docking complex, Neuron 13 (1994) 353–361.
[62] L.A. Swayne, L. Chen, S. Hameed, W. Barr, E. Charlesworth, M.A. Colicos, G.W. Zamponi, J.E. Braun, Crosstalk between huntingtin and syntaxin 1A regulates N-type calcium channels, Mol. Cell Neurosci. 30 (2005) 339–351.
[63] L.H. Chamberlain, M.E. Graham, S. Kane, J.L. Jackson, V.H. Maier, R.D. Burgoyne, G.W. Gould, The synaptic vesicle protein, cysteine-string protein, is associated with the plasma membrane in 3T3-L1 adipocytes and interacts with syntaxin 4, J. Cell Sci. 114 (2001) 445–455.
[64] G.J. Evans, M.C. Wilkinson, M.E. Graham, K.M. Turner, L.H. Chamberlain, R.D. Burgoyne, A. Morgan, Phosphorylation of cysteine string protein by protein kinase A. Implications for the modulation of exocytosis, J. Biol. Chem. 276 (2001) 47877–47885.
[65] J.E. Braun, D.V. Madison, A novel SNAP25-caveolin complex correlates with the onset of persistent synaptic potentiation, J. Neurosci. 20 (2000) 5997–6006.

[66] Z.H. Sheng, J. Rettig, M. Takahashi, W.A. Catterall, Identification of a syntaxin-binding site on N-type calcium channels, Neuron 13 (1994) 1303–1313.
[67] C. Leveque, O. el Far, N. Martin-Moutot, K. Sato, R. Kato, M. Takahashi, M.J. Seagar, Purification of the N-type calcium channel associated with syntaxin and synaptotagmin. A complex implicated in synaptic vesicle exocytosis, J. Biol. Chem. 269 (1994) 6306–6312.
[68] C.T. Yokoyama, S.J. Myers, J. Fu, S.M. Mockus, T. Scheuer, W.A. Catterall, Mechanism of SNARE protein binding and regulation of Cav2 channels by phosphorylation of the synaptic protein interaction site, Mol. Cell Neurosci. 28 (2005) 1–17.
[69] C.T. Yokoyama, Z.H. Sheng, W.A. Catterall, Phosphorylation of the synaptic protein interaction site on N-type calcium channels inhibits interactions with SNARE proteins, J. Neurosci. 17 (1997) 6929–6938.
[70] K.G. Miller, M.D. Emerson, J.R. McManus, J.B. Rand, RIC-8 (Synembryn): a novel conserved protein that is required for G(q)alpha signaling in the *C. elegans* nervous system, Neuron 27 (2000) 289–299.