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Molecular determinants of cysteine string protein modulation of N-type calcium channels

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Summary

Cysteine string proteins (CSPs) are secretory vesicle chaperones that are important for neurotransmitter release. We have previously reported an interaction of CSP with both heterotrimeric GTP-binding proteins (G proteins) and N-type calcium channels that results in a tonic G protein inhibition of the channels. In this report we directly demonstrate that two separate regions of CSP associate with G proteins. The N-terminal binding site of CSP, which includes the J domain, binds G_{α} subunits but not $G_{\beta\gamma}$ subunits whereas the C terminal binding site of CSP associates with either free $G_{\beta\gamma}$ subunits or $G_{\beta\gamma}$ in complex with G_{α} . The interaction of either binding site of CSP (CSP₁₋₈₂ or CSP₈₃₋₁₉₈) with G proteins elicits robust tonic inhibition of N-type calcium channel activity.

However, CSP₁₋₈₂ inhibition and CSP₈₃₋₁₉₈ inhibition of calcium channels occur through distinct mechanisms. Calcium channel inhibition by CSP₈₃₋₁₉₈ (but not CSP₁₋₈₂) is completely blocked by co-expression of the synaptic protein interaction site (synprint) of the N-type channel, indicating that CSP₈₃₋₁₉₈ inhibition is dependent on a physical interaction with the calcium channel. These results suggest that distinct binding sites of CSP can play a role in modulating G protein function and G protein inhibition of calcium channels.

Key words: Cysteine string protein, Chaperones, G proteins, N-type-calcium channels, Synaptic transmission, J domain

Introduction

CSPs (cysteine string proteins) are secretory vesicle chaperone proteins that are evolutionarily conserved. Deletion of CSP in *Drosophila* is semi-lethal; only 4% of the flies develop into adulthood (Zinsmaier et al., 1994). Adult survivors exhibit uncoordinated motor behavior that progresses to paralysis. Recordings from mutant neuromuscular junctions reveal that neurotransmission is reduced by 50% at 22°C and completely abolished above 29°C, indicating that the function of CSP is critical. Spontaneous vesicle release in these CSP-null mutants is not temperature sensitive (Umbach et al., 1994; Saitoe et al., 2001). CSPs have three domains: an N-terminal J domain, a linker-domain and a cysteine string domain. The J domain of CSP is a 70 amino-acid region homologous to the well-characterized bacterial chaperone protein DnaJ and many otherwise unrelated eukaryotic proteins. Although CSP is thought to be important in synaptic transmission, the exact details regarding the role of this synaptic chaperone in neurotransmission are not yet defined. Conflicting reports support either (i) a role for CSP in exocytosis or (ii) a role for CSP in the regulation of transmembrane Ca^{2+} fluxes (reviewed by Zinsmaier and Bronk, 2001) (Chamberlain and Burgoyne, 2000).

We have recently shown that CSP is capable of binding to both the N-type calcium channel and to $G_{\beta\gamma}$ in vitro and that the interaction between CSP and the N-type calcium channel results in a robust tonic inhibition of channel activity by G protein $\beta\gamma$ subunits (Magga et al., 2000). The CSP/G protein interaction was confirmed by co-immunoprecipitation, GST

pull-down assays, crosslinking in intact brain slices as well as evaluation of functional proteins in HEK cells (Magga et al., 2000) and has recently been confirmed by others (Evans et al., 2001). Numerous synaptic proteins were absent from the CSP immunoprecipitations and GST pull-down assays, demonstrating the specificity of the CSP/G protein interaction. Interestingly, CSP and G proteins have been shown to co-enrich in detergent-insoluble lipid raft fractions from rat hippocampus (Magga et al., 2002). Binding of G proteins appears to involve two separate regions of CSP, such that G_{α} interacts with the J domain of CSP in an ATP-dependent manner, whereas $G_{\beta\gamma}$ associates with full-length CSP but not the J domain of CSP in an ATP-independent fashion. Although CSP interacts with G proteins, it is not clear exactly how CSP affects G protein function. In particular, it is unclear exactly which regions of CSP associate with G protein subunits. Furthermore, it is unknown whether CSP's interaction with G_{α} proteins is direct or requires an additional component. Understanding the nature of the interaction between CSP, G proteins and calcium channels is crucial in understanding the molecular role of CSP. In the present study we have therefore analyzed the association of CSP with G proteins and N-type calcium channels.

Materials and Methods

Preparation of rat hippocampal homogenate

Rat hippocampi were hand homogenized with a teflon-coated

homogenizer in 0.32 M sucrose, 10 mM HEPES KOH (pH 7.0), 1 mM EGTA, 0.1 mM EDTA, 0.5 mM PMSF, protease inhibitor cocktail (Boehringer Mannheim), 1 μ M microcystin, 1 μ M okadaic acid and 1 mM sodium orthovanadate (2 ml per hippocampus). The homogenate was centrifuged for 10 minutes at 500 *g* and the supernatant collected and subsequently centrifuged for 20 minutes at 20,000 *g* (4°C). The pellet, containing the synaptic proteins was resuspended in 1% TritonX100, 20 mM MOPS (pH 7.0), 4.5 mM Mg(CH₃COO)₂, 150 mM KCl and 0.5 mM PMSF, protease inhibitor cocktail (Boehringer Mannheim), 1 μ M microcystin, 1 μ M okadaic acid, 1 mM sodium orthovanadate and incubated for 30 minutes at 37°C. Following solubilization large membrane fragments were removed by centrifugation at 1000 *g* for 5 minutes. The resulting supernatant is a crude hippocampal homogenate that contains synaptic proteins. Protein concentrations were determined by Bio-Rad Protein Assay using bovine serum albumin as the standard. All procedures were carried out in strict accordance with a protocol approved by the University of Calgary Animal Care Committee.

Preparation of fusion proteins

GST fusion proteins of CSP and the J domain were prepared as described previously (Braun et al., 1996; Braun and Scheller, 1995). CSP deletion mutants were prepared by subcloning CSP PCR fragments into pGEX-KG (Guan and Dixon, 1991) and expressed as GST fusion proteins in AB1899 cells. His₆ α_{1B} calcium channel II-III linker synprint motif fusion protein (amino acids 718-963) was prepared as described previously (Jarvis et al., 2000). The sequences of all constructs were verified by sequencing both strands using the dideoxynucleotide chain termination method. After induction of expression with 100 μ M isopropyl- β -D-thiogalactopyranoside for 5 hours, the bacteria were suspended in phosphate buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) supplemented with 0.05% Tween 20, 2 mM EDTA and 0.1% β -mercaptoethanol and lysed by two passages through a French Press (Spectronic Instruments, Rochester, NY). The fusion proteins were recovered by binding to glutathione agarose beads (Sigma) or Ni²⁺NTA agarose (Qiagen). The fusion protein beads were washed extensively and finally resuspended in 0.2% TritonX100, 20 mM MOPS (pH 7.0), 4.5 mM Mg(CH₃COO)₂, 150 mM KCl and 0.5 mM PMSF. Recombinant CSP or CSP truncation mutants were purified from the GST fusion protein by cleavage with 0.2 μ M thrombin in 50 mM Tris pH 8, 150 mM NaCl, 2.5 mM CaCl₂, followed by incubation in 0.3 mM PMSF. Synprint was eluted from Ni²⁺NTA agarose with 500 mM imidazole. The protein concentration of recombinant proteins was estimated by Coomassie Blue staining of protein bands after SDS-polyacrylamide gel electrophoresis using bovine serum albumin as a standard.

Immunoblotting

Proteins were transferred electrophoretically at constant voltage from polyacrylamide gels to nitrocellulose (0.45 μ m or 0.2 μ m) in 20 mM Tris, 150 mM glycine, 12% methanol. Transferred proteins were visualized by staining with Ponceau S. Nitrocellulose membranes were blocked for non-specific binding using 5% milk, 0.1% Tween 20, PBS solution [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄ (pH 7.3)] and incubated overnight at 4°C or 2 hours at room temperature with primary antibody. The membranes were washed three to four times in the above milk/Tween/PBS solution and incubated for 30 minutes with goat anti-rabbit or goat anti-mouse IgG-coupled horseradish peroxidase. Antigen was detected using chemiluminescent horseradish peroxidase substrate (ECL, Amersham). Immunoreactive bands were visualized following exposure of the membranes to Amersham Hyperfilm-MP.

Transient transfection of HEK cells

N-type calcium channel subunits, G protein subunits, the α_{1B} II-III linker (corresponding to residues 718-963) and the C-terminus of β ARK (corresponding to residues 495-689) were prepared as described previously (Maggia et al., 2000). cDNAs encoding the entire open reading frame of CSP, CSP₁₋₈₂, CSP₁₋₁₁₂, or CSP₈₃₋₁₉₈ were obtained by polymerase chain reaction (CSP accession number U39320). Sequences were verified and subcloned into pmt2-SX for expression. Human embryonic kidney tsa-201 cells were grown in standard DMEM (Dulbecco's modified Eagle's medium), supplemented with 10% fetal bovine serum and penicillin-streptomycin. The cells were grown to 85% confluency, split with trypsin-EDTA and plated on glass coverslips at 10% confluency 12 hours before transfection. Immediately prior to transfection, the medium was exchanged, and a standard Ca²⁺ phosphate protocol was used to transfect the cells with cDNAs encoding Ca²⁺ channel subunits (α_{1B} + α_2 - δ + β_{1B} ; accession numbers α_{1B} : M92905, α_2 - δ : NM000722, β_{1B} : AB054985) and, as appropriate green fluorescent protein (EGFP; Clontech, CA) CSP, CSP₁₋₈₂ or CSP₈₃₋₁₉₈. After 12 hours, the cells were washed with fresh DMEM and allowed to recover for 12 hours.

Patch clamp recordings

Immediately prior to recording, individual coverslips were transferred to a 3 cm culture dish containing recording solution comprised of 20 mM BaCl₂, 1 mM MgCl₂, 10 mM HEPES, 40 mM Tetraethylammonium chloride (TEA-Cl), 10 mM glucose and 65 mM CsCl, (pH 7.2 with TEA-OH). Whole cell patch clamp recordings were performed using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) linked to a personal computer equipped with pCLAMP v 6.0. Patch pipettes (Sutter borosilicate glass, BF150-86-15) were pulled using a Sutter P-87 microelectrode puller, fire polished using a Narashige microforge, and showed typical resistances of 3 to 4 M Ω . The internal pipette solution contained 108 mM CsMS, 4 mM MgCl₂, 9 mM EGTA, 9 mM HEPES (pH 7.2). Data were filtered at 1 kHz and recorded directly onto the hard drive of the computer. Series resistance and capacitance were compensated. Currents were evoked by stepping from -100 mV to a test potential of +20 mV. The degree of tonic voltage-dependent G protein inhibition was assessed by the degree of current facilitation that occurred after application of a 50 ms depolarizing prepulse to +150 mV, 5 ms prior to the test depolarization. The degree of prepulse relief of the G-protein inhibition was determined by the ratio of the peak current obtained in the absence and presence of the depolarizing prepulse ($I_{\text{peak}} (+pp/-pp)$). The raw data were analyzed using Clampfit and SigmaPlot (Jandel Scientific) software. All figures were generated using SigmaPlot v 4.0. Unless stated otherwise, all error bars are standard errors, and numbers in parentheses displayed in the figures reflect numbers of experiments. Statistical analysis was carried out using SigmaStat 2.0 (Jandel Scientific). Differences between mean values from each group were tested using ANOVAS followed by a Tukey post-ANOVA test for multiple comparisons. Differences were considered significant if $P < 0.05$.

Results

Binding properties of CSP and G proteins

To establish if the interaction between CSP and G α is direct or indirect, glutathione-S-transferase (GST) fusion proteins consisting of full-length CSP or the N-terminus of CSP (amino acids 1-82, including the J domain) coupled to glutathione agarose beads were used in an in vitro binding assay. The beads were incubated with hippocampal homogenate or purified G $\alpha\beta\gamma$, and washed and bound proteins were eluted. The

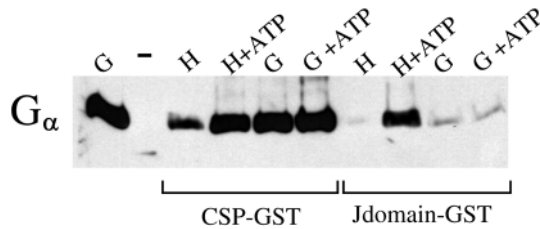


Fig. 1. CSP interacts with G_{α} indirectly. Immunoblot analysis showing binding of G_{α} to CSP-GST fusion protein or J domain GST-fusion protein immobilized on agarose. Fusion proteins were incubated in the presence of hippocampal homogenate (H) or purified $G_{\alpha\beta\gamma}$ proteins (Calbiochem) (G). The beads were washed, and bound proteins were eluted in sample buffer, fractionated by SDS-PAGE and subjected to western blot analysis. The nitrocellulose membrane was probed with an anti- G_{α} polyclonal antibody. These results are representative of five independent experiments.

presence of G_{α} was analyzed by western blotting with an anti- G_{α} polyclonal antibody. Fig. 1 shows that G_{α} from the homogenate as well as the purified $G_{\alpha\beta\gamma}$ preparation associated with full-length CSP in the absence and presence of ATP. In agreement with our previous report (Magga et al., 2000), G_{α} from hippocampal homogenate was observed to interact with the J domain of CSP in the presence of ATP. This G_{α} -J domain interaction was independent of G_{β} , which did not associate with the J domain. In contrast to homogenate G_{α} , the interaction of G_{α} from the purified $G_{\alpha\beta\gamma}$ preparation with the J domain was substantially reduced in the presence of ATP compared to homogenate. The absence of a robust interaction of G_{α} from the purified $G_{\alpha\beta\gamma}$ preparation with the J domain suggests that an additional component present in the homogenate fraction is required for the J-domain- G_{α} association.

Next we examined the nucleotide dependence of the association of G_{α} with the J domain (residues 1-82) of CSP. CSP₁₋₈₂-GST was immobilized on glutathione agarose beads and incubated with equal amounts of hippocampal homogenate in the presence of various nucleotides. Unbound protein was washed away, and the presence of G_{α} , G_{β} and Hsc70 were evaluated by western blot analysis. Fig. 2 shows that the association of G_{α} with immobilized recombinant J domain-GST in vitro was dependent on ATP and that ATP γ S, ADP, GTP, GTP γ S and GDP did not support J-domain- G_{α} -protein association. The presence of G_{β} was analyzed by western blotting with an anti- G_{β} monoclonal. G_{β} did not associate with the J domain under any of the conditions examined. We have previously shown that CSP interacts with and activates the ATPase activity of Hsc70 (Braun et al., 1996). Hsc70 is an abundant neural protein with coupled protein binding and ATPase activities. Although the function of the CSP-Hsc70 complex is unknown, regulation of the assembly/disassembly of multimeric complexes, such as presynaptic complexes, is typical of this family of chaperone proteins. In contrast to the J-domain- G_{α} association, ATP was not essential for the J-domain-Hsc70 interaction (Fig. 2). However, ATP, ADP and GTP increased the association of Hsc70 with CSP in agreement with previous reports (Magga et al., 2000). Overall, these results indicate that the indirect interaction between G_{α} and the J domain of CSP is dependent on ATP binding and that the J domain cannot bind G_{β} .

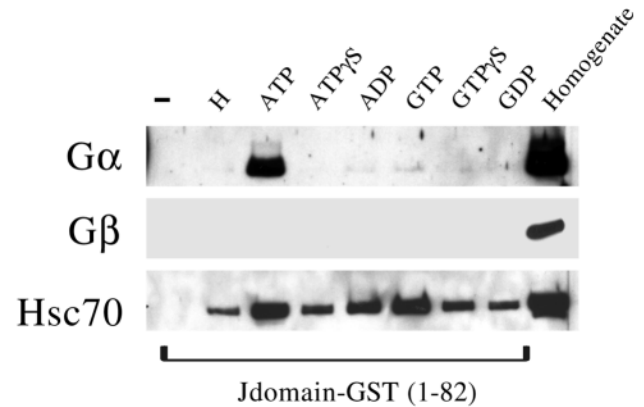


Fig. 2. Nucleotide dependency of G protein interaction with recombinant CSP and J domain. Immunoblot analysis showing binding of G_{α} , G_{β} and Hsc70 to J-domain-GST fusion protein immobilized on agarose. Fusion proteins were incubated in the absence (-) or presence of rat hippocampal homogenate and 2 mM ATP, ATP γ S, ADP, GTP, GTP γ S or GDP at 37°C for 30 minutes. Lane 2 shows fusion proteins incubated with hippocampal homogenate in the absence of nucleotides. Lane 9 shows 15 μ g of hippocampal homogenate loaded directly on the gel. The beads were washed, and bound proteins were eluted in sample buffer, fractionated by SDS-PAGE and subjected to western blot analysis. The nitrocellulose membrane was probed with anti- G_{α} polyclonal, anti- G_{β} monoclonal and anti-Hsc70 monoclonal antibodies. G_{β} does not interact with the J domain in the absence or presence of any nucleotide tested. G_{α} binds to the J domain in the presence of ATP. These results are representative of six independent experiments.

To investigate the stability of the association between G proteins and CSP, we examined their in vitro association over a range of pH. GST fusion proteins consisting of full-length CSP or the N-terminus of CSP (amino acids 1-82, including the J domain) coupled to glutathione agarose beads were used in our in vitro binding assay. Fig. 3 shows that G_{α} and G_{β} associated with full-length CSP. However deletion of amino acids 83-198 resulted in a loss of direct G_{α} and G_{β} binding under all pH conditions examined (in the absence of ATP). Note that at a pH of 3.0 or 10 the recombinant fusion proteins only partially remained immobilized to the agarose beads. The G-protein-CSP complex was detected over the full pH range examined. These observations emphasize that the nucleotide-independent direct interaction between CSP and G proteins is a very stable complex.

CSP binding regions for G proteins and N-type calcium channels

To begin to understand the structural requirements for CSP association with G proteins, a series of CSP deletion mutants were constructed, expressed and purified (Fig. 4). The regions of CSP required for interaction with G proteins were determined through binding experiments with the CSP deletion mutants. Fig. 4C shows the Coomassie staining profile of the CSP-GST truncation mutants. Each of the CSP constructs produced a protein that migrated at the expected molecular weight when analyzed by SDS PAGE with the exception of CSP₈₃₋₁₉₈ and CSP₁₃₇₋₁₉₈. The anomalous migration of these regions of CSP by SDS-PAGE is in agreement with other

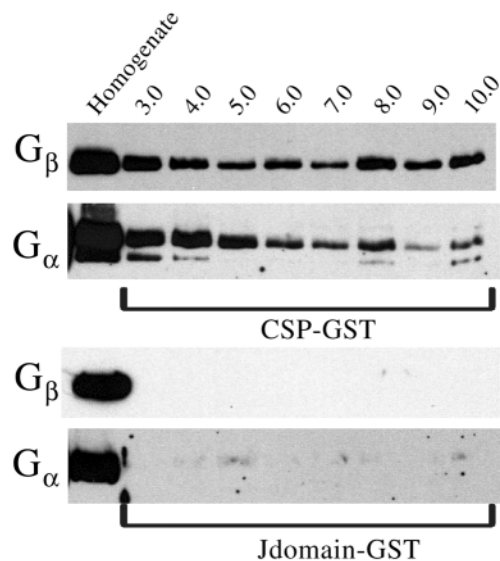


Fig. 3. pH dependency of G protein association with CSP. Immunoblot analysis showing binding of G β and G α to CSP-GST and Jdomain-GST immobilized on agarose. Fusion proteins were incubated with hippocampal homogenate (in the absence of ATP) at the indicated pH for 1 hour. The beads were washed, and bound proteins were eluted in sample buffer, fractionated by SDS-PAGE and subjected to western blot analysis. The nitrocellulose membrane was probed with anti-G β monoclonal and anti-G α polyclonal (Calbiochem). These results are representative of five experiments. G β binding to CSP is very stable over a broad pH range. In contrast, G β does not bind to the J domain under any of the conditions examined.

reports of specific CSP truncations (Evans et al., 2001). To assay G protein binding, the CSP-GST fusion proteins were immobilized on glutathione beads and incubated with equal amounts of hippocampal homogenate. Unbound proteins were washed away and bound proteins were then denatured and fractionated by SDS-PAGE and detected by western blotting. An interesting pattern of binding was revealed through this analysis. Full-length CSP, CSP₈₃₋₁₉₈, CSP₁₋₁₉₀, CSP₁₋₁₈₀, CSP₁₋₁₆₅ and CSP₁₋₁₄₆, were observed to bind to G β in the absence of ATP. In contrast, CSP₁₃₇₋₁₉₈ and CSP₁₋₈₂ did not associate with G β . Addition of residues 83-112 to the J domain of CSP dramatically increased binding of G β , indicating that G β binds to CSP in a region between amino acids 83 and 112. Some association of G β was also observed with the cysteine string region of CSP that is thought to be post-translationally modified *in vivo*; however, such modifications may make G protein association with this region less significant.

The ability of the CSP constructs to support G α binding was also examined. The association of G α with the CSP truncation constructs was not robust; however, weak associations were identified with full-length CSP, CSP₈₃₋₁₉₈, CSP₁₋₁₉₀, CSP₁₋₁₈₀, CSP₁₋₁₆₅ and CSP₁₋₁₄₆. In addition to the identification of hippocampal G α , anti-G α polyclonal (Calbiochem) was observed to crossreact nonspecifically with abundant proteins, especially CSP₈₃₋₁₉₈ and CSP₁₃₇₋₁₉₈ (Fig. 4B). However, anti-G α_i polyclonal (Santa Cruz Inc) confirmed the association of G α with CSP but it did not crossreact with the recombinant proteins, suggesting that the G α that interacts with CSP in the rat hippocampus is a G α_i isoform. The finding that two

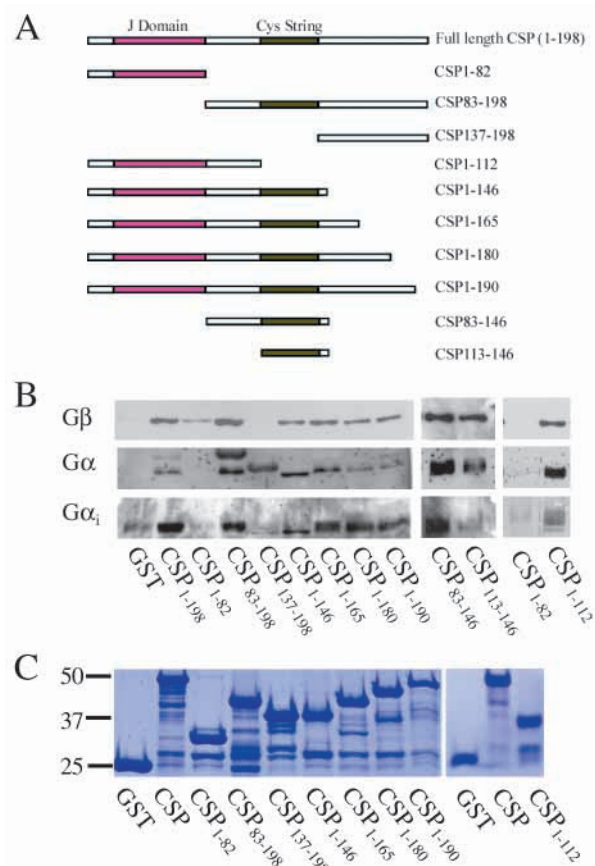


Fig. 4. Identification of interacting domains of CSP and G proteins. (A) Schematic representation of CSP and its deletion mutants encoded by the GST fusion cDNA constructs. (B) These fusion proteins were immobilized on glutathione-agarose beads and incubated with homogenate at 37°C for 30 minutes. The beads were washed, and bound proteins were eluted in sample buffer, fractionated by SDS-PAGE and subjected to western blot analysis. The nitrocellulose membrane was probed with anti-G β monoclonal and anti-G α polyclonal (Calbiochem) and anti-G α_i polyclonal (Santa Cruz) antibodies. (C) Coomassie staining profile of CSP-GST and its truncation mutants. Note that CSP truncation mutants 83-198 and 137-198 migrate anomalously on SDS-PAGE. These results are representative of four independent experiments.

independent antibodies detect association of G α with CSP confirms the G α -CSP interaction. Relatively minor amounts of G α or G β were observed to associate with GST alone compared to CSP, demonstrating the specificity of the CSP-G protein interaction. Taken together our data demonstrate that the N-terminal binding site of CSP, which includes the J domain, binds to G α subunits but not G $\beta\gamma$ subunits, whereas the C terminal binding site of CSP associates with either free G $\beta\gamma$ subunits or G $\beta\gamma$ in complex with G α .

The CSP deletion mutants were also evaluated for their association with α_{1B} His₆ synprint region (amino acids 718-963) of the N-type calcium channel (Fig. 5). To assay synprint binding, the CSP-GST deletion mutants were immobilized on glutathione agarose beads and then incubated with soluble His₆ synprint followed by several washes to remove unbound protein. The proteins were then denatured and fractionated by SDS-PAGE, and bound synprint was detected by western

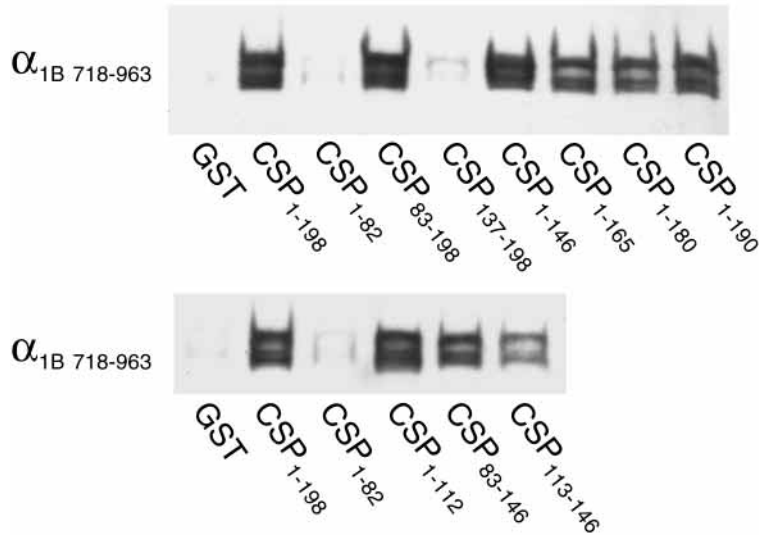


Fig. 5. Identification of the CSP regions that associate with N-type calcium channels. CSP deletion mutants were immobilized on glutathione-agarose beads and incubated with recombinant α_{1B} synprint His₆ at 37°C for 1 hour. The beads were washed, and bound proteins were eluted in sample buffer, fractionated by SDS-PAGE and subjected to western blot analysis. The monoclonal antibody used recognizes a sequence Thr-Leu-Tyr-Asp-Asp-Asp-Lys (Anti Express, Invitrogen) in the fusion proteins. These results are representative of 10 independent experiments.

blotting. Full-length CSP, CSP₈₃₋₁₉₈, CSP₁₋₁₉₀, CSP₁₋₁₈₀, CSP₁₋₁₆₅, CSP₁₋₁₄₆ and CSP₁₋₁₁₂ were observed to bind synprint. Several His₆ fusion proteins in addition to synprint were evaluated and observed not to interact with CSP (data not shown), which demonstrates the specificity of the CSP-synprint association. Synprint did not associate with CSP₁₋₈₂ or CSP₁₃₇₋₁₉₈. Deletion of CSP residues 83-112 corresponding to the 'linker' region significantly reduced synprint's association with CSP, indicating the importance of amino acids 83-112 for the CSP-synprint interaction. Like that found for G_{α} and G_{β} , some association of synprint was observed with the cysteine string region of CSP that is thought to be post-translationally modified *in vivo*. Overall, the binding profile indicates that amino acids between 83-112 of CSP are important for binding synprint, G_{α} and G_{β} subunits.

Two distinct domains of CSP trigger G protein inhibition of N-type channels

We have previously shown that co-expression of Cav2.2 N-type (α_{1B} + α_2 - δ + β_{1b}) calcium channels with CSP in tsa-201 cells results in a tonic inhibition of channel activity by G protein $\beta\gamma$ subunits, which can be reversed by application of strong depolarizing prepulses (Magga et al., 2000). This effect was antagonized by the C-terminal fragment of the β adrenergic receptor kinase, a known $G\beta\gamma$ -binding protein and by overexpression of the CSP-binding region on the N-type calcium channel. On the basis of this evidence, we proposed that CSP acts as a chaperone to promote N-type calcium channel-G=protein interactions. Since the cysteine string region appears to interact with both the N-type calcium channel domain II-III linker and G_{β} (i.e. Figs 4 and 5), we hypothesized that co-expression of this region with N-type calcium channels should mimic the ability of full-length CSP to promote G protein inhibition of N-type calcium channels. As shown in Fig. 6, this is indeed the case. Whereas N-type channels undergo little prepulse facilitation in the absence of coexpressed CSP, coexpression of the cysteine string (CSP₈₃₋₁₉₈) induces tonic modulation that can be reversed by application of a +150 mV voltage pulse, resulting in a 65% increase in peak current amplitude (Fig. 6). These data are consistent with our previous model suggesting that CSP may anchor and chaperone G protein $\beta\gamma$ subunits to the N-type calcium channel (Magga et al., 2000).

By contrast, based on the inability of the J domain (CSP₁₋₈₂) to interact with the channel or $\beta\gamma$ subunits, one might expect co-expression of this region to be ineffective in mediating N-

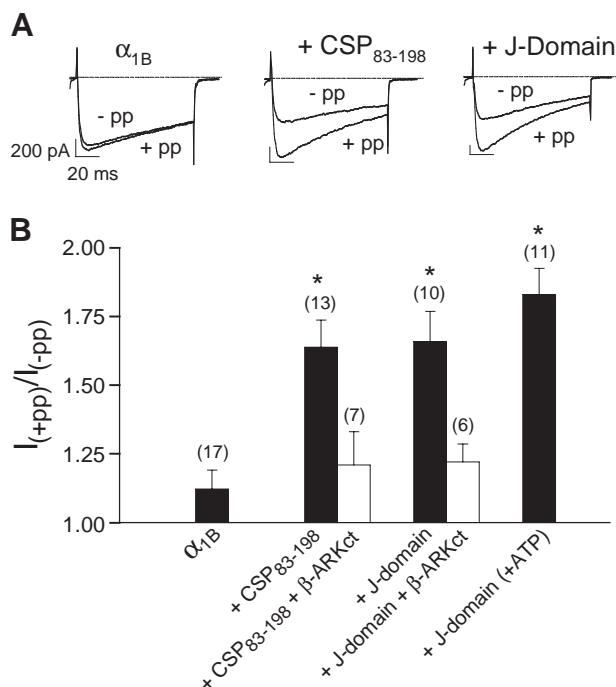


Fig. 6. Regulation of N-type channel activity by individual CSP regions. (A) Current records obtained with N-type (α_{1B} + α_2 - δ + β_{1b}) calcium channels expressed in tsa-201 cells before and after application of a 50 millisecond prepulse (pp) to +150 mV. Currents were elicited by stepping from a holding potential of -100 mV to a test potential of +20 mV. In the absence of CSP (left traces), the prepulses do not affect peak current amplitude. Following coexpression of either the cysteine string domain (middle traces) or the J domain (right traces), the channels undergo a tonic G protein inhibition that is reversed by the prepulses. In all traces, the vertical and horizontal bars indicate, respectively, a current amplitude of 200 pA and a 20 millisecond duration. (B) Bar graph illustrating the degree of prepulse relief in the absence and presence of CSP fragments. Additional coexpression of the C-terminal fragment (residues 495-689) of the β -adrenergic receptor kinase (β ARK) reduces the CSP-mediated current inhibition as shown by the reduced PP relief. The numbers in parentheses reflect the number of experiments; the asterisks indicate statistical significance relative to control conditions at $P < 0.05$ level.

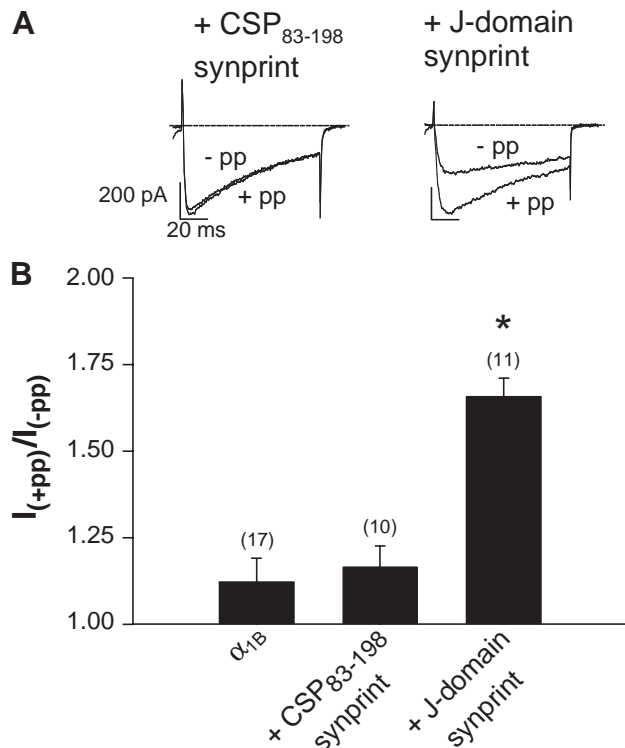


Fig. 7. Effect of synprint peptides on CSP action. (A) Current records obtained with transiently expressed N-type calcium channels following co-expression of either the cysteine string domain (left traces) or the J domain (right traces) and a cDNA construct corresponding to the synprint region of the rat N-type calcium channel. Note that synprint blocks the effect of the cysteine string region but not that mediated by the J domain. The experimental conditions were as outlined in Fig. 6. (B) Bar graphs summarizing the effect of synprint on the G protein effect mediated by the cysteine string and J domain regions. The numbers in parentheses indicate the number of experiments; the asterisk indicates statistical significance relative to control conditions. The data obtained with the J domain in the presence of synprint did not differ significantly from those shown in Fig. 6 in the absence of synprint.

type channel inhibition. To our surprise, however, co-expression of the channel with the J domain resulted in robust prepulse relief, independently of the presence of ATP in the patch pipette (Fig. 6). In addition to CSP₁₋₈₂, co-expression of the channel with CSP₁₋₁₁₂ also resulted in robust prepulse relief (data not shown). To assess whether the CSP₁₋₈₂ and CSP₈₃₋₁₉₈ inhibition of the N-type channel both involved G proteins, we co-expressed N-type channels together with CSP and the C-terminal fragment of the β -adrenergic receptor kinase (β ARK), a known $G_{\beta\gamma}$ -binding protein (Fig. 6). The degree of PP relief was greatly attenuated in the presence of the β ARK fragment, indicating that the CSP-induced N-type calcium channel inhibition is G protein mediated.

If our hypothesis that the cysteine string region serves to colocalize the channel and $G_{\beta\gamma}$ is correct, overexpression of the CSP-binding region on the channel molecule (i.e. the synprint region) should uncouple CSP from the channel and thereby eliminate the G protein effect. As expected, this did indeed occur. As shown in Fig. 7, the ability of the cysteine string domain to induce G protein inhibition of the channel was

virtually abolished following coexpression of the synprint motif. In contrast, the synprint region did not interfere with the J-domain-mediated effect (Fig. 7), indicating that the J-domain-mediated effect occurs independently of the N-type channel domain II-III linker. Hence, separate portions of the CSP molecule can independently promote G protein inhibition of transiently expressed N-type calcium channels, and they can do this by distinct molecular mechanisms.

Discussion

In this study we provide evidence that separate regions of CSPs can independently bind to G proteins and promote G protein inhibition of N-type calcium channels via different mechanisms. In vitro binding assays revealed that the N-terminal binding site of CSP binds to G_{α} subunits but not $G_{\beta\gamma}$ subunits, whereas the C-terminal binding site of CSP associates with either free $G_{\beta\gamma}$ subunits or $G_{\beta\gamma}$ complexed with G_{α} . The 30 amino-acid linker region of CSP (residues 83-112) immediately adjacent to the membrane-anchored cysteine string region binds $G_{\beta\gamma}$ and synprint. Although both regions of CSP modulate N type calcium channels by a mechanism involving G proteins, the sequence of events that underlies this modulation remains to be established. We propose that the C-terminus of CSP (CSP₈₃₋₁₉₈), by physically binding to the II-III linker region of the N-type calcium channel subunit and to the $G_{\beta\gamma}$ protein dimer, may help target $G_{\beta\gamma}$ to its site of action on the N-type calcium channel, which is consistent with our previously proposed model (Maggia et al., 2000) and similar models proposed for syntaxin 1A (Jarvis et al., 2000; Jarvis and Zamponi, 2001a; Jarvis and Zamponi, 2001b). The cytoplasmic II-III linker synprint region is a key binding motif of N-type calcium channels (Sheng et al., 1994).

The observation that the J domain (CSP₁₋₈₂) independently triggers G protein inhibition of the channel is somewhat unexpected (Fig. 6). Neither G_{β} nor synprint were observed to associate with the J domain under any conditions examined (Figs 2-5) (Maggia et al., 2000). However, G_{α} subunits were observed to associate with the J domain of CSP in an indirect and ATP-dependent manner. In contrast to that observed for CSP₈₃₋₁₉₈, co-expression of synprint with CSP₁₋₈₂ and N-type calcium channels did not affect the G protein inhibition of the J domain (Fig. 7). This raises the possibility that the J domain might either stimulate the dissociation of the $G_{\alpha\beta\gamma}$ trimer or, alternatively, that the J domain may prevent the assembly of the trimer. In each case, this would result in free $G_{\beta\gamma}$ subunits that would then be available to produce a tonic inhibition of the channel. To discriminate between the alternatives, we perfused J domain peptides into tsa-201 cells expressing N-type channels, but did not observe the development of G protein inhibition over a 10 minute time course (data not shown), indicating that the J domain cannot acutely trigger the activation of the G protein complex. Hence, we favor a model in which the J domain inhibits the assembly of G_{α} to $G_{\beta\gamma}$ subunits. This would also be consistent with the observation that the physiological effects of the J domain did not depend on the presence of ATP in the patch pipette (Fig. 6), since the J-domain- G_{α} interactions would probably already occur before recordings/patch rupture.

The question remains as to whether two separate actions occur with the full-length CSP construct. As we showed

previously (Magga et al., 2000), the effect of CSP on G protein inhibition of N-type calcium channels was antagonized by overexpression of the synprint motif, indicating that a large part of the action of CSP was due to the interaction of the cysteine string region with the calcium channel II-III linker region. Nonetheless, unlike in the case of the CSP₈₃₋₁₉₈, this inhibition was partial, leaving a statistically significant portion of the G protein effect intact (Magga et al., 2000). It is conceivable that the remaining modulation was mediated by the interaction of the J domain with G α .

The identification of specific Hsc70/DnaJ chaperone machines and the molecular events they regulate in vivo remains a central biological question. Compelling evidence indicates that CSP interacts with and activates the ATPase activity of members of the heat-shock family Hsp70 (Braun et al., 1996; Bronk et al., 2001; Chamberlain and Burgoyne, 1997; Stahl et al., 1999). Recently, SGT (small glutamine rich tetratricopeptide repeat) has been shown to be a key component of the CSP-Hsc70 chaperone machine (Tobaben et al., 2001). Formation of the trimeric CSP-SGT-Hsc70 complex strongly activates the ATPase activity of Hsc70 (Tobaben et al., 2001). Given the number of proteins and protein complexes participating in neurotransmitter release, it is likely that CSP chaperone activity is important in supervising specific transitions and interactions among synaptic proteins, such as G proteins, during the rapid exocytosis/endocytosis synaptic vesicle cycle.

CSP was originally proposed to promote the activity of presynaptic calcium channels (Mastrogriacomo et al., 1994; Gundersen and Umbach, 1992). However, additional lines of evidence have suggested alternative CSP/calcium channel models. Several studies conclude that CSP is important in exocytosis rather than the regulation of calcium transmembrane fluxes (Brown et al., 1998; Chamberlain and Burgoyne, 1998; Zhang et al., 1999; Zhang et al., 1998; Cribbs et al., 1998; Graham and Burgoyne, 2000). In contrast, the synprint sites of P/Q calcium channels (Leveque et al., 1998) and N type calcium channels (Magga et al., 2000) have been shown to bind CSP. Although CSP has been proposed to play a role in the recruitment of calcium channels (Chen et al., 2002), CSP has also been proposed to play an inhibitory role in depolarization dependent calcium entry (Dawson-Scully et al., 2002), which is consistent with our results (Magga et al., 2000). In addition to G proteins (Magga et al., 2000) and calcium channels (Chen et al., 2002; Leveque et al., 1998; Wu et al., 1999; Magga et al., 2000), several other targets of CSP chaperone activity have been proposed including syntaxin (Nie et al., 1999; Evans et al., 2001; Wu et al., 1999), VAMP (also called synaptobrevin) (Leveque et al., 1998), synaptotagmin I (Evans and Morgan, 2002), α GDI (Sakisaka et al., 2002) and CFTR (Zhang et al., 2002). Further experimentation is required to establish the role of CSP in controlling the aggregation state and conformation of these possible target proteins and the nature of the conflicting results regarding the role of CSP in exocytosis.

In conclusion, our results reveal that two separate motifs of CSP bind to G proteins and regulate G protein inhibition of N-type calcium channels. The multitude of G-protein-triggered cascades and the distinct mechanisms of action by CSP N- and C-terminal domains may, in part, explain the paradoxical findings regarding CSP's role in exocytosis. Chaperone-

assisted protein folding of signaling components has been shown for other signal transduction cascades (e.g. transcription factors and glucocorticoid receptors) (reviewed in Hartl and Hayer-Hartl, 2002). The regulation of G protein function by chaperones such as CSP represents an important concept with regard to the control of neurotransmitter release and synaptic efficacy.

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References

- Braun, J. E. and Scheller, R. H. (1995). Cysteine string protein, a DnaJ family member, is present on diverse secretory vesicles. *Neuropharmacology* **34**, 1361-1369.
- Braun, J. E., Wilbanks, S. M. and Scheller, R. H. (1996). The cysteine string secretory vesicle protein activates Hsc70 ATPase. *J. Biol. Chem.* **271**, 25989-25993.
- Bronk, P., Wenniger, J. J., Dawson-Scully, K., Guo, X., Hong, S., Atwood, H. L. and Zinsmaier, K. E. (2001). *Drosophila* Hsc70-4 is critical for neurotransmitter exocytosis in vivo. *Neuron* **30**, 475-488.
- Brown, H., Larsson, O., Branstrom, R., Yang, S., Leibiger, B., Leibiger, I., Fried, G., Moede, T., Deeney, J. T., Brown, G. R. et al. (1998). Cysteine string protein (CSP) is an insulin secretory granule-associated protein regulating beta-cell exocytosis. *EMBO J.* **17**, 5048-5058.
- Chamberlain, L. H. and Burgoyne, R. D. (1997). Activation of the ATPase activity of heat-shock proteins Hsc70/Hsp70 by cysteine-string protein. *Biochem. J.* **322**, 853-858.
- Chamberlain, L. H. and Burgoyne, R. D. (1998). Cysteine string protein functions directly in regulated exocytosis. *Mol. Cell. Biol.* **9**, 2259-2267.
- Chamberlain, L. H. and Burgoyne, R. D. (2000). Cysteine string protein: the chaperone at the synapse. *J. Neurochem.* **74**, 1781-1789.
- Chen, S., Zheng, X., Schulze, K. L., Morris, T., Bellen, H. and Stanley, E. F. (2002). Enhancement of presynaptic calcium current by cysteine string protein. *J. Physiol.* **538**, 383-389.
- Cribbs, L. L., Lee, J. H., Yang, J., Satin, J., Zhang, Y., Daud, A., Barclay, J., Williamson, M. P., Fox, M., Rees, M. and Perez-Reyes, E. (1998). Cloning and characterization of α 1H from human heart, a member of the T-type Ca²⁺ channel gene family. *Circ. Res.* **83**, 103-109.
- Dawson-Scully, K., Bronk, P., Atwood, H. L. and Zinsmaier, K. E. (2002). Cysteine-string protein increases the calcium sensitivity of neurotransmitter exocytosis in *Drosophila*. *J. Neurosci.* **20**, 6039-6047.
- Evans, G. J., Wilkinson, M. C., Graham, M. E., Turner, K. M., Chamberlain, L. H., Burgoyne, R. D. and Morgan, A. (2001). Phosphorylation of cysteine string protein by protein kinase A. Implications for the modulation of exocytosis. *J. Biol. Chem.* **276**, 47877-47885.
- Evans, G. J. O. and Morgan, A. (2002). Phosphorylation-dependent interaction of the synaptic vesicle protein cysteine string protein and synaptotagmin. *Biochem. J.* **364**, 343-347.
- Graham, M. E. and Burgoyne, R. D. (2000). Comparison of cysteine string protein (CSP) and mutant α -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**, 1281-1289.
- Guan, K. L. and Dixon, J. E. (1991). Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal. Biochem.* **192**, 262-267.
- Gundersen, C. B. and Umbach, J. A. (1992). Suppression cloning of the cDNA for a candidate subunit of a presynaptic calcium channel. *Neuron* **9**, 527-537.
- Hartl, F. U. and Hayer-Hartl, M. (2002). Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* **295**, 1852-1858.
- Jarvis, S. E. and Zamponi, G. W. (2001a). Distinct molecular determinants

- govern syntaxin 1A-mediated inactivation and g-protein inhibition of N-type calcium channels. *J. Neurosci.* **21**, 2939-2948.
- Jarvis, S. E. and Zamponi, G. W. (2001b). Interactions between presynaptic Ca²⁺ channels, cytoplasmic messengers and proteins of the synaptic vesicle release complex. *Trends Pharmacol. Sci.* **10**, 519-525.
- Jarvis, S. E., Magga, J. M., Beedle, A. M., Braun, J. E. and Zamponi, G. W. (2000). Modulation of N-type calcium channels by G β -gamma is enhanced by syntaxin. *J. Biol. Chem.* **275**, 6388-6394.
- Leveque, C., Pupier, S., Marqueze, B., Geslin, L., Kataoka, M., Takahashi, M., de Waard, M. and Seagar, M. (1998). Interaction of cysteine string proteins with the α 1A subunit of the P/Q-type calcium channel. *J. Biol. Chemistry* **273**, 13488-13492.
- Magga, J. M., Jarvis, S. E., Arnot, M. I., Zamponi, G. W. and Braun, J. E. (2000). Cysteine string protein regulates G-protein modulation of N-type calcium channels. *Neuron* **28**, 195-204.
- Magga, J. M., Kay, J. G., Davy, A., Poulton, N. P., Robbins, S. M. and Braun, J. E. A. (2002). ATP dependence of the SNARE/caveolin1 interaction in the hippocampus. *Biochem. Biophys. Res. Commun.* **291**, 1232-1238.
- Mastrogiacono, A., Parsons, S. M., Zampighi, G. A., Jenden, D. J., Umbach, J. A. and Gunderson, C. B. (1994). Cysteine string proteins: a potential link between synaptic vesicles and presynaptic Ca²⁺ channels. *Science* **263**, 981-982.
- Nie, Z., Ranjan, R., Wenniger, J. J., Hong, S. N., Bronk, P. and Zinsmaier, K. E. (1999). Overexpression of cysteine-string proteins in *Drosophila* reveals interactions with syntaxin. *J. Neurosci.* **19**, 10270-10279.
- Saitoe, M., Schwartz, T. L., Umbach, J. A., Gunderson, C. B. and Kidokoro, Y. (2001). Absence of junctional glutamate receptor clusters in *Drosophila* mutants lacking spontaneous transmitter release. *Science* **293**, 514-517.
- Sakisaka, T., Meerlo, T., Matteson, J., Plutner, H. and Balch, W. E. (2002). Rab-alphaGDI activity is regulated by a Hsp90 chaperone complex. *EMBO J.* **21**, 6125-6135.
- Sheng, Z. H., Rettig, J., Takahashi, M. and Catterall, W. A. (1994). Identification of a syntaxin-binding site on N-type calcium channels. *Neuron* **13**, 1303-1313.
- Stahl, B., Tobaben, S. and Sudhof, T. C. (1999). Two distinct domains in hsc70 are essential for the interaction with the synaptic vesicle cysteine string protein. *Eur. J. Cell Biol.* **78**, 375-381.
- Tobaben, S., Thakur, P., Fernandez-Chacon, R., Sudhof, T. C., Rettig, J. and Stahl, B. (2001). A trimeric protein complex functions as a synaptic chaperone machine. *Neuron* **31**, 987-999.
- Umbach, J. A., Zinsmaier, K. E., Eberle, K. K., Buchner, E., Benzer, S. and Gunderson, C. B. (1994). Presynaptic dysfunction in *Drosophila* csp mutants. *Neuron* **13**, 899-907.
- Wu, M. N., Fergestad, T., Lloyd, T. E., He, Y., Broadie, K. and Bellen, H. J. (1999). Syntaxin 1A interacts with multiple exocytic proteins to regulate neurotransmitter release in vivo. *Neuron* **23**, 593-605.
- Zhang, H., Kelley, W. L., Chamberlain, L. H., Burgoyne, R. D., Wollheim, C. B. and Lang, J. (1998). Cysteine-string proteins regulate exocytosis of insulin independent from transmembrane ion fluxes. *FEBS Lett.* **437**, 267-272.
- Zhang, H., Kelley, W. L., Chamberlain, L. H., Burgoyne, R. D. and Lang, J. (1999). Mutational analysis of cysteine-string protein function in insulin exocytosis. *J. Cell Sci.* **112**, 1345-1351.
- Zhang, H., Peters, K. W., Sun, F., Marino, C. R., Lang, J., Burgoyne, R. D. and Frizzell, R. A. (2002). Cysteine string protein interacts with and modulates the maturation of CFTR. *J. Biol. Chem.* **277**, 28948-28958.
- Zinsmaier, K. E. and Bronk, P. (2001). Molecular chaperones and the regulation of neurotransmitter exocytosis. *Biochem. Pharmacol.* **62**, 1-11.
- Zinsmaier, K. E., Eberle, K. K., Buchner, E., Walter, N. and Benzer, S. (1994). Paralysis and early death in cysteine string protein mutants of *Drosophila*. *Science* **263**, 977-980.