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\(_\alpha\) 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\(_\alpha\). 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, 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 (Maggi 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 (Maggi 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 (Maggi et al., 2002). Binding of G proteins appears to involve two separate regions of CSP, such that G\(_\alpha\) 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\(_\alpha\) 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 μM microcystin, 1 μM 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 μM microcystin, 1 μM 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 Glu1 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 μM isopropyl-β-D-thiogalactopyranoside for 5 hours, the bacteria were suspended in phosphate buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 2.7 mM KH2PO4, 10 mM Na2HPO4, 150 mM KCl and 0.5 mM PMSF, protease inhibitor cocktail (Boehringer Mannheim), 1 μM microcystin, 1 μM 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.

Immunoblotting

Proteins were transferred electrophoretically at constant voltage from polyacrylamide gels to nitrocellulose (0.45 μm or 0.2 μm) 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 α1β II-III linker (corresponding to residues 718-963) and the C-terminus of βARK (corresponding to residues 495-689) were prepared as described previously (Magga 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 pm2-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 (α1β + α2-δ + β1c, accession numbers α1B: M92905, α2-δ: NM000722, β1C: 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-CI), 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 MΩ. 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 (Ipeak (+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 Gαi 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 Gαi1, and washed and bound proteins were eluted. The
GTP, GTP
GST in vitro was dependent on ATP and that ATP
and incubated with equal amounts of hippocampal homogenate
domain cannot bind G
J domain of CSP is dependent on ATP binding and that the J
domain–G-protein–CSP complex was detected over the full pH range
Note that at a pH of 3.0 or 10 the recombinant fusion proteins
acids 83-198 resulted in a loss of direct G
b binding
presence of Gα was analyzed by western blotting with an anti-
Gα polyclonal antibody. Fig. 1 shows that Gα from the
homogenate as well as the purified Gαβγ preparation associated with full-length CSP in the absence and presence of ATP. In agreement with our previous report (Magga et al., 2000), Gα from hippocampal homogenate was observed to interact with the J domain of CSP in the presence of ATP. This Gα-J domain interaction was independent of Gβ, which did not associate with the J domain. In contrast to homogenate Gα, the interaction of Gα from the purified Gαβγ preparation with the J domain was substantially reduced in the presence of ATP compared to homogenate. The absence of a robust interaction of Gα from the purified Gαβγ preparation with the J domain suggests that an additional component present in the homogenate fraction is required for the J-domain–Gα association.

Next we examined the nucleotide dependency of the association of Gα 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 Gα, Gβ and Hsc70 were evaluated by western blot analysis. Fig. 2 shows that the association of Gα with immobilized recombinant J-domain-GST in vitro was dependent on ATP and that ATPγS, ADP, GTP, GTPγS and GDP did not support J-domain–G-protein association. The presence of Gβ was analyzed by western blotting with an anti-Gβ monoclonal. Gβ 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–Gα 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 Gα and the J domain of CSP is dependent on ATP binding and that the J domain cannot bind Gβ.

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 Gα and Gβ associated with full-length CSP. However deletion of amino acids 83-198 resulted in a loss of direct Gα and Gβ 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 CSPs3-198 and CSPs137-198. The anomalous migration of these regions of CSP by SDS-PAGE is in agreement with other
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 subjected to western blot analysis. The nitrocellulose membrane was probed with anti-Gβ monoclonal and anti-Gα polyclonal (Calbiochem). These results are representative of five experiments. Gβ binding to CSP is very stable over a broad pH range. In contrast, Gβ does not bind to the J domain under any of the conditions examined.

independent antibodies detect association of Gα with CSP confirms the Gα-CSP interaction. Relatively minor amounts of Gα or Gb 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 Gα subunits but not Gβ subunits, whereas the C terminal binding site of CSP associates with either free Gβ subunits or Gαβ in complex with Gα.

Fig. 3. pH dependency of G protein association with CSP. Immunoblot analysis showing binding of Gβ and Gα 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-Gβ monoclonal and anti-Gα polyclonal (Calbiochem). These results are representative of five experiments. Gβ binding to CSP is very stable over a broad pH range. In contrast, Gβ does not bind to the J domain under any of the conditions examined.

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-Gβ monoclonal and anti-Gα polyclonal (Calbiochem) and anti-GαI 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.
CSP 1-165, CSP 1-146 and CSP 1-112 were observed to bind shown), which demonstrates the specificity of the CSP-synprint association with CSP, indicating the importance of amino acids between 83-112 of CSP 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\(\alpha\) and G\(\beta\), 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\(\alpha\) and G\(\beta\) subunits.

Separate regions of CSP modulate G proteins

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 (\(\alpha_{1B} + \alpha_2 - \delta + \beta_{1B}\)) calcium channels with CSP in tsa-201 cells results in a tonic inhibition of channel activity by G protein \(\beta y\) 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 \(\beta\) adrenergic receptor kinase, a known \(G_{\beta y}\)-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\(\beta\) (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 (CSP\(_{83-198}\)) 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 \(\beta y\) subunits to the N-type calcium channel (Magga et al., 2000).

By contrast, based on the inability of the J domain (CSP\(_{1-82}\)) to interact with the channel or \(\beta y\) subunits, one might expect co-expression of this region to be ineffective in mediating N-
Inhibition is G protein mediated. indicating that the CSP-induced N-type calcium channel was greatly attenuated in the presence of the (data not shown). To assess whether the CSP1-82 and CSP83-198 domain to induce G protein inhibition of the channel was occur. As shown in Fig. 7, the ability of the cysteine string 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 Gα subunits but not Gβγ subunits, whereas the C-terminal binding site of CSP associates with either free Gβγ subunits or Gβγ complexed with Gα. The 30 amino-acid linker region of CSP (residues 83-112) immediately adjacent to the membrane-anchored cysteine string region binds Gβγ 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 Gβγ protein dimer, may help target Gβγ to its site of action on the N-type calcium channel, which is consistent with our previously proposed model (Magga 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 Gα nor synprint were observed to associate with the J domain under any conditions examined (Figs 2-5) (Magga et al., 2000). However, Gα 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 Gαβγ trimer or, alternatively, that the J domain may prevent the assembly of the trimer. In each case, this would result in free Gβγ 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 GαGβγ 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–Gα 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 Go.

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 (Mastrogiacomo 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), aCGD (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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