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 ($CSP_{1\text{-}82}$ or $CSP_{83\text{-}198}$) with G proteins elicits robust tonic inhibition of N-type calcium channel activity. However, $CSP_{1\text{-}82}$ inhibition and $CSP_{83\text{-}198}$ inhibition of calcium channels occur through distinct mechanisms. Calcium channel inhibition by $CSP_{83\text{-}198}$ (but not $CSP_{1\text{-}82}$) is completely blocked by co-expression of the synaptic protein interaction site (synprint) of the N-type channel, indicating that $CSP_{83\text{-}198}$ 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 βγ 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 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 $Mg(CH_3COO)_2$, 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. His_6 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 μM isopropyl-β-D-thiogalactopyranside for 5 hours, the bacteria were suspended in phosphate buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) 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 Ni^{2+}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 $Mg(CH_3COO)_2$, 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 μM thrombin in 50 mM Tris pH 8, 150 mM NaCl, 2.5 mM $CaCl_2$, followed by incubation in 0.3 mM PMSF. Synprint was eluted from Ni^{2+}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 eletrophoretically 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 Na_2HPO_4, 1.4 mM KH_2PO_4 (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 βARK (corresponding to residues 495-689) were prepared as described previously (Magga et al., 2000). cDNAs encoding the entire open reading frame of CSP, $CSP_{1\text{-}82}$, $CSP_{1\text{-}112}$, or $CSP_{83\text{-}198}$ 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 Ca^{2+} phosphate protocol was used to transfect the cells with cDNAs encoding Ca^{2+} channel subunits (α_{1B} + α_2-δ + $\beta_{1b;}$ accession numbers α_{1B}: M92905, α_2-δ: NM000722, β_{1b}: AB054985) and, as appropriate green fluorescent protein (EGFP; Clontech, CA) CSP, $CSP_{1\text{-}82}$ or $CSP_{83\text{-}198}$. 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 $BaCl_2$, 1 mM $MgCl_2$, 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 MΩ. The internal pipette solution contained 108 mM CsMS, 4 mM $MgCl_2$, 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 ($I_{peak\ (+pp/-pp)}$). 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 comparisions. 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

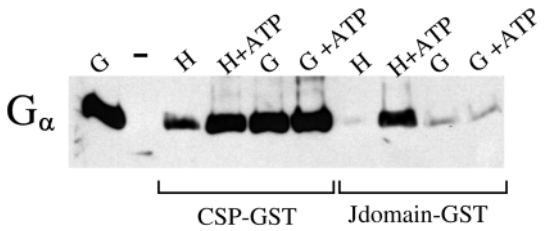

Fig. 1. CSP interacts with G_α indirectly. Immunoblot analysis showing binding of G_α to CSP-GST fusion protein or J domain GST-fusion protein immobilized on agarose. Fusion proteins were incubated in the presence of hippocampal homogenate (H) or purified $G_{\alpha\beta\gamma}$ proteins (Calbiochem) (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.

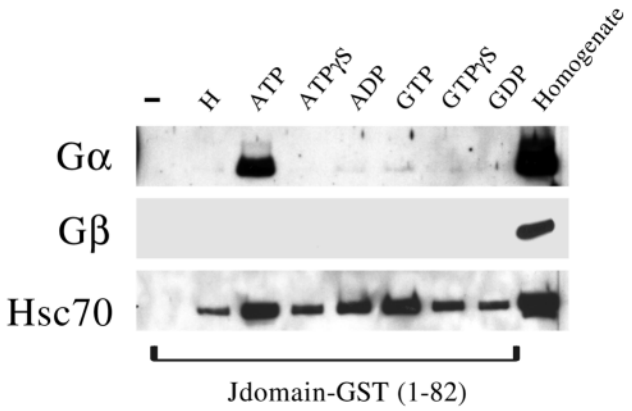

Fig. 2. Nucleotide dependency of G protein interaction with recombinant CSP and J domain. Immunoblot analysis showing binding of G_α, G_β and Hsc70 to J-domain–GST fusion protein immobilized on agarose. Fusion proteins were incubated in the absence (–) or presence of rat hippocampal homogenate and 2 mm ATP, ATPγS, ADP, GTP, GTPγS 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 μg 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.

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. $CSP_{1\text{-}82}$-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 ATPγS, ADP, GTP, GTPγS and GDP did not support J-domain–G-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.

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 $CSP_{83\text{-}198}$ and $CSP_{137\text{-}198}$. The anomalous migration of these regions of CSP by SDS-PAGE is in agreement with other

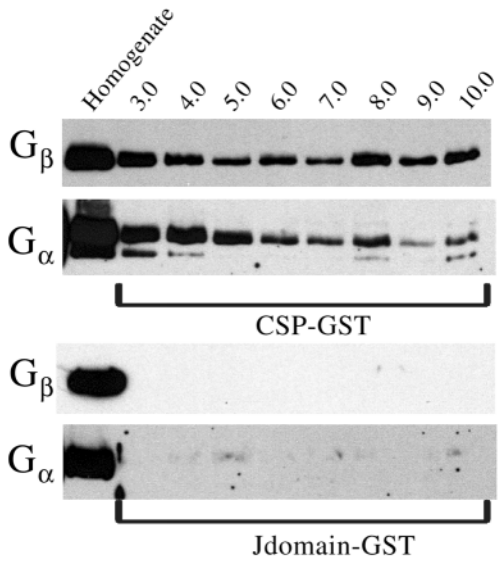

Fig. 3. pH dependency of G protein association with CSP. Immunoblot analysis showing binding of Gβ and Gα to CSP-GST and Jdomain-GST immobilized on agarose. Fusion proteins were incubated with hippocampal homogenate (in the absence of ATP) at the indicated pH for 1 hour. The beads were washed, and bound 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.

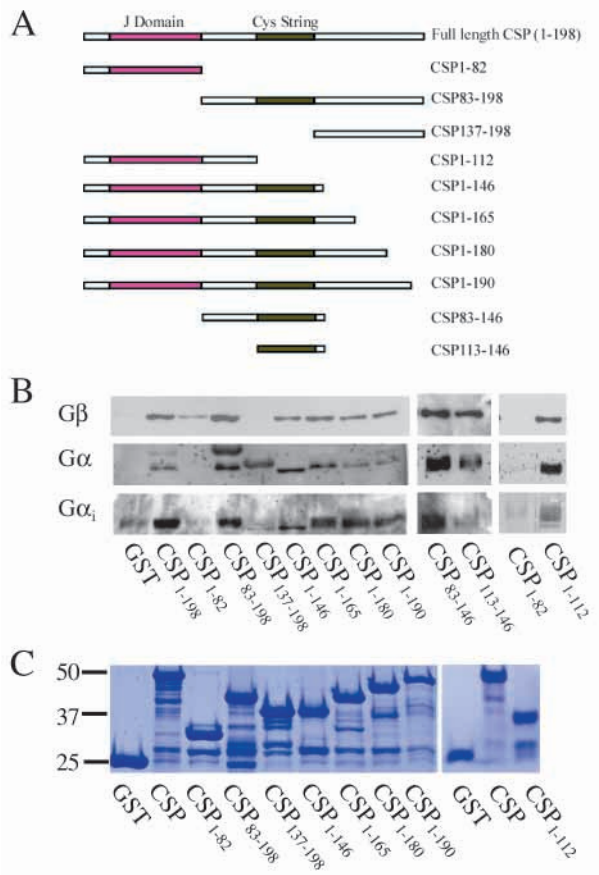

Fig. 4. Identification of interacting domains of CSP and G proteins. (A) Schematic representation of CSP and its deletion mutants encoded by the GST fusion cDNA constructs. (B) These fusion proteins were immobilized on glutathione-agarose beads and incubated with homogenate at 37°C for 30 minutes. The 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.

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, $CSP_{83\text{-}198}$, $CSP_{1\text{-}190}$, $CSP_{1\text{-}180}$, $CSP_{1\text{-}165}$ and $CSP_{1\text{-}146}$, were observed to bind to Gβ in the absence of ATP. In contrast, $CSP_{137\text{-}198}$ and $CSP_{1\text{-}82}$ did not associate with Gβ. Addition of residues 83-112 to the J domain of CSP dramatically increased binding of Gβ, indicating that Gβ binds to CSP in a region between amino acids 83 and 112. Some association of Gβ 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 Gα binding was also examined. The association of Gα with the CSP truncation constructs was not robust; however, weak associations were identified with full-length CSP, $CSP_{83\text{-}198}$, $CSP_{1\text{-}190}$, $CSP_{1\text{-}180}$, $CSP_{1\text{-}165}$ and $CSP_{1\text{-}146}$. In addition to the identification of hippocampal Gα, anti-Gα polyclonal (Calbiochem) was observed to crossreact nonspecifically with abundant proteins, especially $CSP_{83\text{-}198}$ and $CSP_{137\text{-}198}$ (Fig. 4B). However, anti-Gαi polyclonal (Santa Cruz Inc) confirmed the association of Gα with CSP but it did not crossreact with the recombinant proteins, suggesting that the Gα that interacts with CSP in the rat hippocampus is a Gαi isoform. The finding that two independent antibodies detect association of Gα with CSP confirms the Gα-CSP interaction. Relatively minor amounts of Gα or Gβ 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 Gβγ subunits, whereas the C terminal binding site of CSP associates with either free Gβγ subunits or Gβγ in complex with Gα.

The CSP deletion mutants were also evaluated for their association with α_{1B} His_6 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 His_6 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

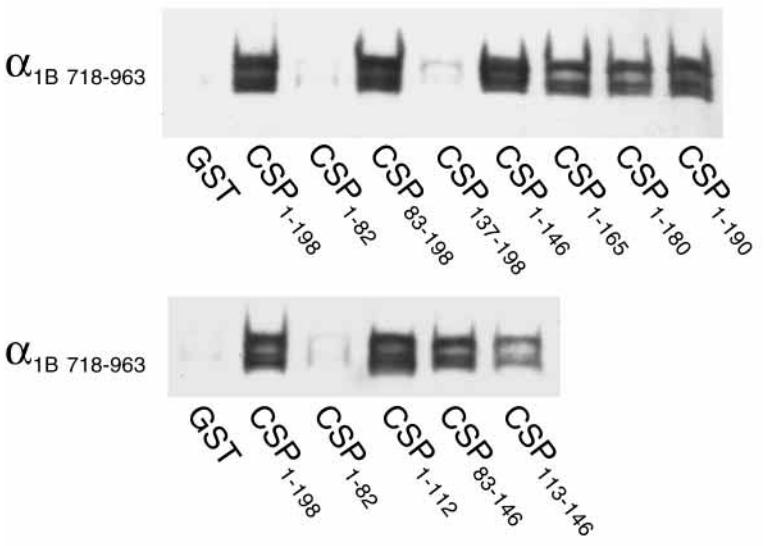

Fig. 5. Identification of the CSP regions that associate with N-type calcium channels. CSP deletion mutants were immobilized on glutathione-agarose beads and incubated with recombinant α_{1B} synprint His_6 at 37°C for 1 hour. The beads were washed, and bound proteins were eluted in sample buffer, fractionated by SDS-PAGE and subjected to western blot analysis. The monoclonal antibody used recognizes a sequence Thr-Leu-Tyr-Asp-Asp-Asp-Asp-Lys (Anti Express, Invitrogen) in the fusion proteins. These results are representative of 10 independent experiments.

blotting. Full-length CSP, CSP_{83-198}, CSP_{1-190}, CSP_{1-180}, CSP_{1-165}, CSP_{1-146} and CSP_{1-112} were observed to bind synprint. Several His_6 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 CSP_{1-82} or $CSP_{137-198}$. 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 Cav.2.2 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 $\beta\gamma$ 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 (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 $\beta\gamma$ 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 $\beta\gamma$ subunits, one might expect co-expression of this region to be ineffective in mediating N-

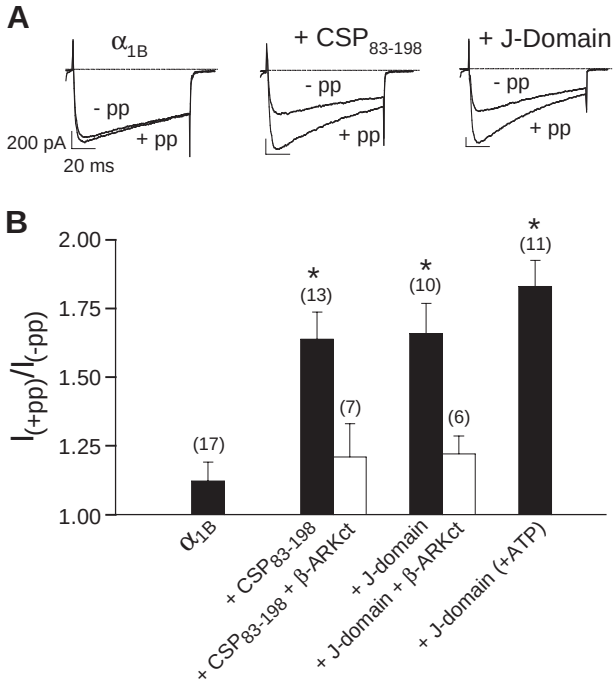

Fig. 6. Regulation of N-type channel activity by individual CSP regions. (A) Current records obtained with N-type ($\alpha_{1B} + \alpha_2 - \delta + \beta_{1b}$) calcium channels expressed in tsa-201 cells before and after application of a 50 millisecond prepulse (pp) to +150 mV. Currents were elicited 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 milliseond 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 (βARK) 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.

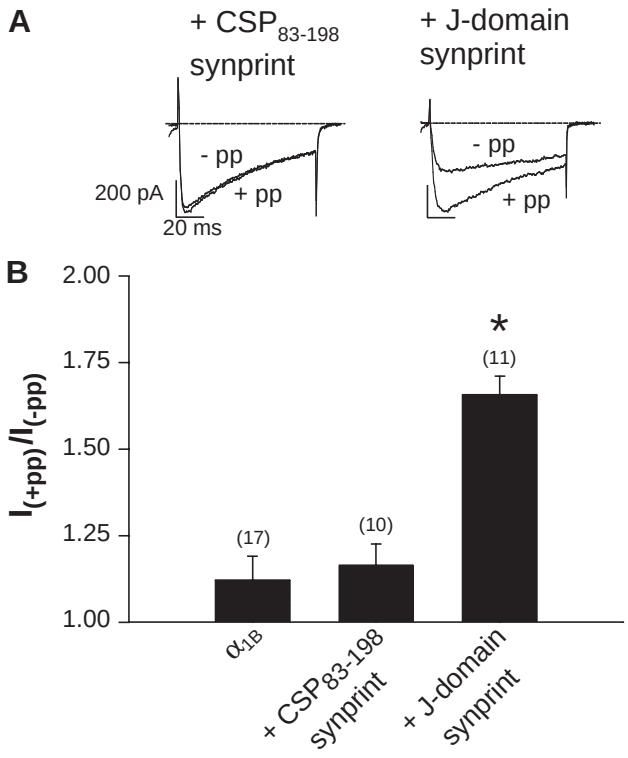

Fig. 7. Effect of synprint peptides on CSP action. (A) Current records obtained with transiently expressed N-type calcium channels following co-expression of either the cysteine string domain (left traces) or the J domain (right traces) and a cDNA construct corresponding to the synprint region of the 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 CSP_{1-82}, co-expression of the channel with CSP_{1-112} also resulted in robust prepulse relief (data not shown). To assess whether the CSP_{1-82} and CSP_{83-198} 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 (βARK), a known $G_{\beta\gamma}$-binding protein (Fig. 6). The degree of PP relief was greatly attenuated in the presence of the βARK 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 (CSP_{83-198}), 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 (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 (CSP_{1-82}) 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) (Magga 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 CSP_{83-198}, co-expression of synprint with CSP_{1-82} 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 CSP_{83-198}, 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 (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), αGDI (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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