Interaction of Cysteine String Proteins with the α1A Subunit of the P/Q-type Calcium Channel*

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Cysteine string proteins (Csps) are J-domain chaperone proteins anchored at the surface of synaptic vesicles. Csps are involved in neurotransmitter release and may modulate presynaptic calcium channel activity, although the molecular mechanisms are unknown. Interactions between Csps, proteins of the synaptic core (SNARE) complex, and P/Q-type calcium channels were therefore explored. Co-immunoprecipitation suggested that Csps occur in complexes containing synaptobrevin (VAMP), but not syntaxin 1, SNAP-25, nor P/Q-type calcium channels labeled with 125I-ω-conotoxin MVIIIC. However binding experiments with 35S-labeled Csp1 demonstrated an interaction (apparent KD = 700 nM at pH 7.4 and 4 °C) with a fusion protein containing a segment of the cytoplasmic loop linking homologous domains II-III of the α1A calcium channel subunit (BI isoform, residues 780–969). Binding was specific as it was displaced by unlabeled Csp1, and no interactions were detected with fusion proteins containing other calcium channel domains, VAMP, or syntaxin 1A. A Csp binding site on the P/Q-type calcium channel is thus located within the 200 residue synaptic protein interaction site that can also bind syntaxin I, SNAP-25, and synaptotagmin I. Csp may act as a molecular chaperone to direct assembly or disassembly of exocytotic complexes at the calcium channel.

The association of Csps with synaptic vesicles (7, 10) suggests that they are involved in membrane trafficking and/or exocytosis of neurotransmitters. Deletion of the cspg gene in Drosophila causes temperature-sensitive failure of synaptic transmission, resulting in paralysis (13) due to impaired excitation-secretion coupling at nerve terminals (14, 15). Recent findings suggest that the default involves either a deficit in calcium entry or in the ability of calcium to trigger exocytosis (16). These data are thus consistent with earlier results indicating that Csps act as positive modulators of ω-conotoxin GVIA-sensitive calcium channels from Torpedo electric organ, heterologously expressed in Xenopus oocytes (2). Fusion competent synaptic vesicles are thought to be docked at the active zone in close proximity to the voltage-gated calcium channels that trigger release. It has been proposed that interactions between calcium channels and Csps may be necessary for the channels associated with docked vesicles to open in response to depolarization (7), although biochemical evidence in favor of this interaction has not been reported. We have therefore examined the molecular interactions of Csp with proteins of the synaptic core (SNARE) complex (17) and P/Q-type calcium channels, which support a major fraction of transmitter release in many synaptic fields of the mammalian brain (18).

EXPERIMENTAL PROCEDURES

Recombinant Proteins—A maltose-binding protein (MBP)-Csp1 fusion protein was prepared (10) and purified by affinity chromatography on an amylose-Sepharose column (New England Biolabs). Glutathione S-transferase (GST) fusion proteins containing domains of the rabbit brain calcium channel α1A (BI-2 isoform) subunit (19), syntaxin 1A, VAMP 2, and synaptotagmin IV were generated as reported previously (20, 21). A GST fusion protein, including sequence from the III-IV loop of α1A, was constructed by amplifying base pairs 4561–4725 and subcloning into pGEXKG. Fusion protein production was induced in cultures of the protease-deficient Escherichia coli strain BL21, and lysates were purified with glutathione-agarose. 35S-Csp1 was synthesized by coupled transcription and translation in vitro, in the presence of [35S]cysteine (>600 Ci/mmol, DuPont), using the TNT™ system (Promega).

Immunoprecipitation Assays—Polyclonal antibodies against MBP-Csp1 (10) were purified on protein A-Sepharose Fast Flow beads (Amersham Pharmacia Biotech). Immunoprecipitation of 35S-Csp1 labeled P/Q-type calcium channels from CHAPS extracts of a rat cerebellar P2 fraction was performed as described previously (22). 35S-Csp1 was incubated for 2 h at 4 °C with 10 μg of anti-Csp antibodies in Triton-X-buffered saline (TBS) containing 0.3% (w/v) BSA. 35S-Csp1antibody complexes were then recovered by mixing with protein A-Sepharose Fast Flow beads for 1 h at 4 °C, centrifuging for 30 s at 10000 × g, and then washed three times in TBS, 0.3% BSA. The washed pellet was resuspended in 10 ml of scintillation solvent, and 35S-Csp binding was quantified by scintillation counting.

Binding Assays—Binding assays were performed by incubating GST fusion proteins with in vitro-translated 35S-Csp in 300 μl of TBS containing 0.5% BSA at 4 °C in the presence of 30 μl glutathione-agarose beads (20, 21). Beads were washed three times by centrifugation with
FIG. 1. Co-immunoprecipitation of cysteine string proteins and VAMP. Rat brain homogenates were solubilized in 1% CHAPS, 0.32 M sucrose, 1 mM EDTA, 10 mM Heps/NaOH pH 7.4, cleared by filtration (0.22-μm pore), and incubated with antibodies or control IgG, in the presence or absence of MBP-Csp. Immune complexes were recovered on protein A-Sepharose beads, and Csp, syntaxin 1, SNAP-25, and VAMP were detected by Western blotting. The lane labeled “Total” was loaded with 1% of the amount of protein subjected to immunoprecipitation assay in the other lanes.

The same buffer, and 35S-Csp binding was quantified by scintillation counting.

Rat brain homogenates were solubilized in 1% of the indicated detergent (see figure legends) in 10 mM Heps, 0.15 M NaCl, 2 mM MgCl2, 1 mM EGTA, adjusted to pH 7.4 with NaOH, and incubated for 5 h at 4 °C with GST or GST fusion proteins bound to glutathione-agarose beads. Beads were then washed once with TBS containing 0.3% BSA and once with TBS, and bound Csp was detected by SDS-PAGE and immunoblotting (10).

Surface Plasmon Resonance Spectroscopy—Spectroscopy was performed on a BIAcore apparatus (Pharmacia Biosensor), with GST fusion proteins immobilized on the sensor chip via covalently linked anti-GST antibodies. 2 μM MBP-Csp or MBP were introduced at a flow rate of 5 μl/min at 22 °C.

RESULTS

Experiments were performed in order to explore the molecular mechanisms by which Csps may modulate exocytosis. First we performed co-immunoprecipitation experiments to examine the hypothesis that Csps may associate with components of the trimeric synaptic core (SNARE) complex and/or presynaptic P/Q-type calcium channels.

Co-immunoprecipitation Experiments Reveal Protein Complexes Containing Csp and VAMP—Rat brain membranes were solubilized with CHAPS, and detergent extracts were incubated with non-immune immunoglobulins, polyclonal antibodies raised against Csp in the absence and presence of MBP-Csp, and polyclonal anti-VAMP antibodies. Immune complexes were recovered on protein A-Sepharose beads, and bound Csp was detected by SDS-PAGE and immunoblotting.

Binding of 35S-Csp to the II-III Linker Region of the Calcium Channel α1A Subunit—35S-Csp1 was produced by coupled transcription and translation in vitro in the presence of [35S]cysteine. SDS-PAGE and autoradiography revealed a single major radioactive species migrating at 28 kDa for Csp1, and 21 kDa for a polypeptide from the calcium channel II-III linker region corresponding to residues 780–969 of the α1A isomer of the P/Q calcium channel (22). As reported previously prelabeled channels were immunoprecipitated by the monoclonal anti-synaptobrevin 1 antibody 10H5 (Fig. 2) (22). In contrast anti-Csp antibodies failed to capture solubilized 125I-α-conotoxin MVIIIC receptors. The amount of radioactivity immunoprecipitated by anti-Csp antibodies was not significantly different from that in control assays in which anti-Csp antibodies were preincubated with a large excess of MBP-Csp1 or in which non-immune rabbit IgG was used. As this approach did not detect interactions between endogenous Csps and P/Q-type calcium channels, experiments were performed with recombinant proteins.

Binding of 35S-Csp to the II-III Linker Region of the Calcium Channel α1A Subunit—35S-Csp1 was produced by coupled transcription and translation in vitro in the presence of [35S]cysteine. SDS-PAGE and autoradiography revealed a single major radioactive species migrating at 28 kDa in accordance with the predicted molecular mass of Csp (Fig. 3A, left panel). Furthermore radioactivity was immunoprecipitated by anti-Csp antibodies but not by non-immune immunoglobulins (Fig. 3A, right panel). SDS-PAGE and protein staining indicated that the predominant forms of MBP-Csp1, MBP, GST-II-III, and GST (Fig. 3B) migrated at 70, 43, 48, and 27 kDa respectively, consistent with apparent molecular masses of 27 kDa for Csp1, and 21 kDa for a polypeptide from the calcium channel II-III linker region corresponding to residues 780–969 of the BI isoform of the α1A subunit.

Binding experiments were then performed in which 35S-Csp1 was incubated with a range of concentrations of GST-II-IIIα or GST. Thus in this assay 35S-Csp1 is considered as being the

![Diagram](Image)
binding site and the GST fusion protein as the ligand. Saturable binding of GST-II-III \(_\alpha\) occurred with an apparent equilibrium dissociation constant of 700 nM (Fig. 3C). Interaction was specific as binding was not detected with an identical concentration range of GST. Furthermore the addition of a large molar excess of MBP-Csp1 inhibited the association of \(^{35}\)S-Csp\(_1\) with the GST-II-III loop (Fig. 3D). No significant binding to \(^{35}\)S-Csp\(_1\) was detected with GST fusion proteins containing VAMP 2, syntaxin 1A, domains from the I-II or III-IV linker regions (Fig. 3D), or from the amino- and carboxyl-terminal regions (not shown) of the \(\alpha_1\)A subunit.

Surface plasmon resonance experiments using a Biacore apparatus to detect interactions between recombinant proteins also confirmed MBP-Csp1 binding to GST-II-III \(_\alpha\) immobilized on a sensor chip via covalently linked anti-GST antibodies (not shown). Binding monitored by this method was again specific for the II-III domain of the \(\alpha_1\)A subunit, as control experiments did not reveal interactions between MBP and GST-II-III \(_\alpha\), nor between MBP-Csp1 and other cytoplasmic domains of the \(\alpha_1\)A subunit fused to GST.

**Interactions between Recombinant and Endogenous Synaptic Proteins**—Preliminary surface plasmon resonance experiments indicated that the binding of Csp to the GST-II-III \(_\alpha\) fusion protein was diminished in the presence of CHAPS. The effect of a series of detergents (digitonin, cholate, Lubrol, Triton X-100, Nonidet P-40, CHAPS) on the interaction of \(^{35}\)S-Csp\(_1\) with the GST-II-III \(_\alpha\) was examined. All the detergents tested (at 1%, w/v) inhibited binding by 60–80% (not shown). As detergent concentrations within this range are required to solubilize native calcium channels from brain membranes, it is possible that dissociation of endogenous Csp/calcium channel complexes by detergents may account for our inability to detect interactions by co-immunoprecipitation (see Fig. 2). In order to examine this possibility the interaction of recombinant calcium channel domains with native Csp was studied. Detergent extracts of P2 membranes from rat brain were incubated with either GST-II-III \(_\alpha\), GST-III-IVA, or GST immobilized on glutathione-Sepharose beads. After washing, beads were recovered by centrifugation, treated with SDS-PAGE sample buffer, and the eluted proteins analyzed by Western blotting with anti-Csp antibodies (Fig. 4A). This approach demonstrated that endogenous Csp associated specifically with the GST-II-III \(_\alpha\) fusion protein despite the presence of CHAPS, Nonidet P-40, or Triton X-100.

Similar experiments were performed to examine whether endogenous P/Q-type calcium channels interact with recombinant Csp. MBP-Csp1 and control proteins were immobilized on amylose beads and incubated under agitation with CHAPS extracts of synaptic membranes containing native calcium channels prelabeled with \(^{125}\)I-\(\omega\)-conotoxin MVIIIC. P/Q-type calcium channels were captured by immobilized anti-syntaxin 1 antibodies, but not by MBP-Csp beads. Furthermore the quantity of solubilized calcium channels trapped by MBP-Csp1 beads was not significantly different from that bound by beads linked to MBP alone or fused to synaptotagmin IV (Fig. 4B).
Detergents at 1%, w/v, and aliquots were mixed with GST-II-IIIA, GST-proteins A. Rat brain homogenates were solubilized in the indicated
pendent assays; III-IVA, or GST bound to glutathione-Sepharose beads. After washing, taxin 1 with P/Q-type channels in
viewed in Refs. 23 and 24). SNARE complexes associate with
membrane: syntaxin 1 and SNAP-25 (17). This complex is
synaptic vesicle protein: synaptobrevin (VAMP) and two pro-
channel. The synaptic core (SNARE) complex is composed of a
the trimeric synaptic core complex and/or the P/Q-type calcium
channels to open in response to membrane depolarization (2,
release of neurotransmitters (13, 14) and suggests that they
may regulate both exocytosis and calcium channel function by
channel gating properties (26). It is therefore possible that Csp
interacting with components of the SNARE complex. Co-immu-
 NOPRECIPITATION experiments revealed complexes containing
Csp and VAMP, but not syntaxin 1 or SNAP-25. Synaptophysin,
which interacts with VAMP in synaptic vesicles (27), was
not identified among proteins immunoprecipitated by anti-Csp
antibodies (not shown). Direct binding between recombinant
Csp1 and VAMP 2 was not detected. However in vitro translated
and bacterially expressed Csps are not palmitoylated, and a direct interaction between Csps and VAMP could require
fatty acylation. These data are thus consistent with either a
direct interaction between native Csp and VAMP or an indirect
association mediated by unidentified partners.

The calcium-conducting pore of P/Q-type calcium channels is
formed by the $\alpha_2\delta$ subunit, which contains four homologous
domains each composed of six helical transmembrane seg-
ments (19). A GST-fusion protein containing a domain located
in the cytoplasmic loop linking homologous domains II and III
of the $\alpha_2\delta$ subunit displayed saturable binding to in vitro
translated $\[^{35}S\]$cysteine-labeled Csp. The affinity of this inter-
action is moderate with an apparent equilibrium dissociation
constant of 700 nM at 4 °C and pH 7.4. Csp thus binds an
approximately 200 residue segment on the $\alpha_2\delta$ subunit (resi-
dues 780–969 of the BI isoform). Specific interaction was con-
firmed using surface plasmon resonance spectroscopy, which
indicated that MBP-Csp can bind to the same GST fusion
protein immobilized on a sensor chip.

In view of the molecular chaperone activity of Csp, it is
important to underline the specificity of binding. Hsp70 and
dnaJ-like proteins act as chaperones and bind polypeptides,
but with different selectivity. Hsp70 binds unfolded proteins
recognizing short stretches of amino acids with an extended
conformation. In contrast, dnaJ-like proteins such as Csp gen-
erally bind substrates exhibiting secondary and tertiary struc-
ture, but exhibit very low affinity for polypeptides in extended
conformations (reviewed in Ref. 28). Our data demonstrate that
recombinant Csp does not associate nonselectively with bacte-
rially expressed proteins irrespective of their sequence, as Csp
did not bind to GST alone, or GST fusion proteins containing
syntaxin 1, VAMP 2, or other regions of the $\alpha_2\delta$ subunit.
Furthermore it is reasonable to assume that the folding of in vitro
translated proteins provides a closer approximation to
native conformation than bacterially expressed proteins fused
to large tag sequences. Although the illustrated binding data
were obtained using in vitro translated Csp and GST-II-IIIA,
we have also verified that in vitro translated $\[^{35}S\]$methionine-
labeled II-III $\alpha$ interacts with MBP-Csp1 (not shown). Thus
specific binding occurs irrespective of which partner is gener-
ated by in vitro translation and which is a fusion protein.
Finally it is significant that Csp binds selectively to a calcium
channel domain strongly implicated in excitation-secretion cou-
pling. This region of the $\alpha_2\delta$ subunit constitutes a binding site
for syntaxin 1 and SNAP-25 (BI residues 722–1036, Ref. 25)
and synaptotagmin I (BI residues 780–969, Ref. 21). The func-
tional relevance of these interactions is supported by evidence
that injection of the equivalent synaptic protein binding domain
from the $\alpha_2B$ subunit (29) disrupts transmitter release in
sympathetic neurons (30) and at the amphibian neuromuscular
junction (31).

In contrast to experiments with recombinant proteins, im-
imunoprecipitation with anti-Csp antibodies failed to capture
P/Q-type calcium channels from detergent extracts of nerve
terminals. This could be due to the masking of Csp epitopes by
protein/protein interactions or alternatively to the disruption of
native complexes during membrane solubilization. All deter-
gents tested inhibited GST-II-IIIA fusion protein binding to
$\[^{35}S\]$-Csp. However the presence of detergents in membrane
extracts did not prevent endogenous Csps from interacting
with GST-II-IIIA fusion proteins. In contrast native calcium

FIG. 4. Interactions between endogenous and recombinant
proteins A. Rat brain homogenates were solubilized in the indicated
detergents at 1%, w/v, and aliquots were mixed with GST-II-IIIA, GST-
III-IVA, or GST bound to glutathione-Sepharose beads. After washing,
bound Csp was detected by Western blotting. B, detergent extracts
containing prelabeled P/Q-type calcium channels were incubated with
protein-A Sepharose beads coupled to anti-syntaxin 1 antibodies or
amylose beads coupled to MBP-Csp, MBP-synaptotagmin IV, or MBP.
Following washing bound calcium channel-radioligand complexes were
quantified by $\gamma$ counting. Results are the mean of at least three inde-
pendent assays; error bars represent + S.D.

DISCUSSION
Evidence points to an essential role for Csps in the synaptic
release of neurotransmitters (13, 14) and suggests that they
are involved in regulating the ability of presynaptic calcium
canals to open in response to membrane depolarization (2,
16). We thus asked whether Csps interact with components of
the trimeric synaptic core complex and/or the P/Q-type calcium
channel. The synaptic core (SNARE) complex is composed of a
synaptic vesicle protein: synaptobrevin (VAMP) and two pro-
teins that are predominantly expressed in the synaptic plasma
membrane: syntaxin 1 and SNAP-25 (17). This complex is
thought constitute the hub of a sequence of protein/protein
interactions that lead up to calcium-dependent exocytosis (re-
viewed in Refs. 23 and 24). SNARE complexes associate with
P/Q-type calcium channels (22, 25) and co-expression of syn-
taxin 1 with P/Q-type channels in Xenopus oocytes modulates
channel gating properties (26). It is therefore possible that Csp
may regulate both exocytosis and calcium channel function by
interacting with components of the SNARE complex. Co-immu-
noprecipitation experiments revealed complexes containing

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channels did not bind to immobilized MBP-Csp, suggesting that a limiting parameter may be the accessibility of the Csp binding site on the calcium channel. Syntaxin 1, SNAP-25, and synaptotagmin I bind to the same domain on the calcium channel as Csp, and these three proteins all display a higher affinity than Csp for binding to calcium channels (25, 21). Furthermore a large fraction of native P/Q-type channels are stably associated with complexes containing syntaxin I, SNAP-25, VAMP, and synaptotagmin I or II (21, 22). SNARE complexes associated with native calcium channels may therefore prevent interaction with endogenous or exogenous Csps.

If Csps promote calcium channel opening by binding to the II-III linker region of the α2δ subunit, how may intrinsic chaperone activity and cooperation with Hsp70 be involved? Apart from their role in protein folding during translation, chaperones contribute to the assembly and dissociation of multi-protein complexes. Hsp70 (DnaK) and J-domain proteins (DnaJ) erone activity and cooperation with Hsp70 be involved? Apart interaction with endogenous or exogenous Csps.

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