The Variable C-Terminus of Cysteine String Proteins Modulates Exocytosis and Protein−Protein Interactions†

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ABSTRACT: Cysteine string proteins (Csps) are vesicle proteins involved in neurotransmission and hormone exocytosis. They are composed of distinct domains: a variable N-terminus, a J-domain followed by a linker region, a cysteine-rich string, and a C-terminus which diverges among isoforms. Their precise function and interactions are not fully understood. Using insulin exocytosis as a model, we show that the linker region and the C-terminus, but not the variable N-terminus, regulate overall secretion. Moreover, endogenous Csp1 binds in a calcium-dependent manner to monomeric VAMP2, and this interaction requires the C-terminus of Csp. The interaction is isoform specific as recombinant Csp1 binds VAMP1 and VAMP7, but not VAMP3. Cross-linking in permeabilized clonal β-cells revealed homodimerization of Csp which is stimulated by Ca^{2+} and again modulated by the variant C-terminus. Our data suggest that both interactions of Csp occur during exocytosis and may explain the effect of the variant C-terminus of this chaperon protein on peptide hormone secretion.

The peptide hormone insulin is stored in large dense core vesicles in pancreatic β-cells and released by exocytosis, a multistage process involving transport of vesicles to the plasma membrane, their docking, their priming, and finally their fusion with the plasma membrane (*1*). The SNARE[1] hypothesis has been proposed as a unifying model to account in molecular terms for membrane docking and/or fusion of exocytotic vesicles in different secretory systems (*2*, *3*). Regulated exocytosis requires proteins in addition to this core complex to ensure controlled hormone or neurotransmitter release coupled to the appropriate stimulus. The cysteine string proteins (Csps) are one of the components involved and probably act as molecular chaperones at a late stage, though their exact function remains unknown (*4*).

Csps were originally identified as synaptic vesicle proteins in *Drosophila* implicated in neurotransmitter release (*5*, *6*). Csps have recently been classified as members of DnaJ homologue subfamily C, and in mammals, several isoforms have been described: Csp1 and Csp2, which are generated by alternative splicing and differ only in their C-terminus (*7*), as well as the predicted isoforms Csp-β and Csp-γ (*4*). Csp1 is expressed on synaptic vesicles and on large dense core vesicles (LDCVs) in mammalian neuroendocrine and in endocrine cells such as the pancreatic β-cells (*7*−*10*). Csps contain several domains: the N-terminal J-domain, which stimulates the ATPase activity of chaperones such as Hsc70 (*11*, *12*), a functionally defined linker region (*13*), and the hydrophobic cysteine string region, containing a dense cluster of 11−15 mostly acylated cysteine residues (*14*). Whereas these domains are highly conserved throughout evolution, the extreme N-terminus (amino acids 1−13) and the C-terminus are variable.

Several proteins interacting with Csp have been described, and some binding regions have been mapped. Csp interacts directly with synaptotagmin I, upon the phosphorylation of a serine at the N-terminus of Csp (*15*), with the P/Q-type Ca^{2+} channels (*16*) and the chaperone Hsp90 (*17*), via its J-domain with Hsc70 (*11*−*13*) and the α-subunit of heterotrimeric G-proteins (*18*). G-Protein β-subunits bind the C-terminal portion of Csp, including the cysteine string and the C-terminus (*19*). Moreover, a trimeric complex of Csp, Hsc70, and the cochaperone SGT has been characterized (small glutamine-rich tetratricopeptide repeat containing protein), wherein SGT interacts with the cysteine string motif (*20*, *21*). Divergent data have been reported for the interaction between Csp1 and the SNARE protein syntaxin (*16*, *22*−*24*).

These interactions have been observed mainly in synaptic vesicle preparations. It is not clear whether they also apply to large dense core vesicles (LDCVs) and whether they occur during Ca^{2+} stimulation of exocytosis in cellular systems. β-cells provide a convenient model as we and others have previously demonstrated the role of Csp in the exocytosis of LDCVs independent of regulation of Ca^{2+} fluxes (*9*, *10*). We have now examined the effect of different truncations

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[1] Abbreviations: Csp, cysteine string protein; DMSO, dimethyl sulfoxide; DSS, disuccinimidyl suberate; GST, glutathione *S*-transferase; hCB, human cellubrevin; LDCV, large dense core vesicle; SNAP-23, synaptosome-associated protein of 23 kDa; SNAP-25, synaptosome-associated protein of 25 kDa; SNARE, soluble NSF attachment receptor; VAMP, vesicle-associated membrane protein.

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on exocytosis and defined two protein−protein interactions. Our results indicate that the variable C-terminus modulates interactions with the SNARE protein VAMP and homodimerization during exocytosis of LDCVs.

EXPERIMENTAL PROCEDURES

Materials. The following reagents were used: glutathione−Sepharose 4B (Phamarcia), protein A−agarose (VectorLab), protein G−sepharose 4B beads and isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma), leupeptin semisulfate and aprotinin (Sigma), lysozyme (Roche), fluorophore-coupled second antibodies (Jackson Laboratories), and TNT *in vitro* transcription and translation system (Promega). All cross-linking reagents were obtained from Pierce. Polyclonal rabbit anti-Csp1 antiserum was raised against recombinant Csp1 (*7*). In addition, the commercial anti-CSP antibody A1567 (Chemicon) directed against soluble domains was used. Csp1 and Csp2 cDNA sequences were cloned from bovine adrenal chromaffin cells and ligated into the *Bam*HI−*Eco*RI sites of pcDNA3 (Invitrogen), pcDNA3-myc, pcDNA3-2myc, or pCMV-Tag (Stratagene). Streptolysin O was prepared and purified as described previously (*25*). cDNAs encoding the cytoplasmic domains of human VAMP3 (amino acids 1−71) and VAMP7 (amino acids 1−179 in pGEX-2T), SNAP-23 (in pcDNA3), and polyclonal rabbit antibody against VAMP7 (*26*) were generously provided by T. Galli (Paris, France). The plasmid encoding GST−VAMP2 (amino acids 1−96) was kindly provided by R. Regazzi (Lausanne, Switzerland). The following monoclonal antibodies were used: anti-VAMP (Synaptic Systems, Goettingen, Germany), anti-syntaxin 1 (anti-HPC-1, Sigma), anti-insulin (K36aC10, Sigma), and anti-synaptophysin (SVP38, Roche). Monoclonal anti-myc antibodies were produced from 9E10 myeloma cell culture medium.

Molecular Cloning. The *Bam*HI−*Eco*RI fragments of wild-type Csp1, wild-type Csp2, and the point mutants Csp1H43Q and Csp1E93V were excised from pcDNA3 and subcloned into the corresponding sites of the pGEX-2T or pQE30-Csps vector. Constructions of Csp point mutants have been described previously (*13*). Csp deletion mutants were constructed by PCR using the following primers: $Csp1_{14-198}$ or $Csp2_{14-167}$ plus strand (TCGGATCCGAGTCCCTGTACCATGTACTGG) and minus strand (ACGAATTCATTTAGTTGAACCCGTCGGTGTG), Csp_{1-82} and Csp_{1-110} plus strand (AAGGATCCATGGCTGACCAGAGGCAGCG), Csp_{1-82} minus strand (AGGAATTCACAGTGAGCCGTACTTGTCG), and Csp $_{1-110}$ minus strand (AGGAATTCACAGGGCCTTGGCCCACCAGC); *Bam*HI or *Eco*RI restriction sites are underlined. The Csp_{1-110} with the E93V mutation was obtained using Csp1E93V as a template. The PCR products were digested with *Bam*HI and *Eco*RI, purified from 1.5% agarose gels, and inserted into the corresponding sites of a pcDNA3-myc vector. Fragments containing $Csp1_{83-198}$ and $Csp1_{137-198}$ were digested from pQE-30 vectors (*12*) and inserted in frame into the pcDNA3-myc vector. All constructs were verified by sequencing of both strands. 2myc-Csp was obtained by excision of myc-Csp1 and its ligation into a myc-epitope encoding pcDNA3 plasmid. Flag-Csp constructs were obtained by in-frame insertion of Csp fragments into the appropriate pCMV-Tag plasmid (Stratagene).

Cell Culture, Transient Transfection, Secretion, and Immunohistochemistry. Cell culture, transient transfection, and secretion assays were performed as described previously (*13*, *27*). For immunofluorescence, HIT-T15 cells were seeded on glass coverslips. Two days later, cells were transfected with the indicated constructs as described previously. Cell permeabilization with streptolysin O, immunocytochemistry, and confocal microscopy were performed as described previously (*13*). Imaging of transiently transfected cells was performed on an LSM 510 Meta confocal laser microscope (Zeiss) with a 100× objective, and quantitative image analysis was carried out using the incorporated software after thresholding and creation of a mask. To determine expression levels of transiently expressed proteins by immunoblots, cells were detached 48 h after transfection by incubation for 5 min at 37 °C with PBS containing 10 mM EDTA, centrifuged at 4 °C and 14000*g* for 10 min, resuspended in ice-cold PBS-EDTA, sonicated, and again centrifuged for 30 min at 4 °C and 14000*g*. Samples were applied on 12% SDS−PAGE or on Tricine gels (*28*) and transferred to a PVDF membrane. Subsequently, blots were incubated with primary antibodies (anti-myc antibody, 1:20 dilution; anti-Csp1 antibody, 1:3000 dilution; AB1576, 1:5000), and antibody binding was detected by chemiluminescence (Roche).

Immunoprecipitation and Binding Assays. Polyclonal anti-Csp1 antibody (20 μL), monoclonal anti-VAMP2 antibody (20 μL), or nonimmunoreactive IgG_1 (20 μL) was immobilized to 100 μL of protein A−Sepharose beads by incubation at 4 °C overnight in intracellular buffer A [140 mM L-glutamic acid/monopotassium salt, 5 mM NaCl, 7 mM $MgSO_4$, and 20 mM HEPES (pH 7)] in the presence of 10 mM EGTA and 0.1 μM free Ca^{2+} (low calcium, LC) or 10 μM free Ca^{2+} (high calcium, HC) (*27*). Subsequently, the antibody-coupled beads were washed three times with buffer A containing 5 mM ATP. Crude membranes (50 μL, 200 μg) from HIT-T15 cells were extracted with 1% Triton X-100 in buffer A in the presence of LC or HC for 1 h, followed by centrifugation at 15000*g* for 1 h. The resulting supernatant was mixed with antibody-coupled protein A−Sepharose beads at 4 °C and incubated overnight on a shaking platform. Subsequently, the mixture was centrifuged at 15000*g* for 5 min, and the pellets were washed three times with 1 mL of intracellular buffer containing the corresponding concentration of Ca^{2+}. Finally, pellets were resuspended in 100 μL of SDS−PAGE sample buffer. After the samples had been heated for 5 min at 100 °C, 60 μL was analyzed by Western blotting probed with antibodies against Csp1, syntaxin, synaptophysin, or VAMP2. All procedures for immunoprecipitation were carried out at 4 °C. Signals were quantified as described in Cross-Linking.

Recombinant glutathione *S*-transferase (GST) or His_6 epitope-tagged fusion proteins (Csp1, Csp2, Csp1H43Q, Csp1E93V, VAMP2, TI-VAMP, hCB, and GST) were purified according to the protocol provided by the manufacturer (Phamacia and Qiagen, Courtaboeuf, France), followed by dialysis for 48 h in buffer A with at least four changes of buffer. Purification of these proteins was verified by Coomassie blue staining of 12% SDS−PAGE gels, and the specificity of recombinant GST−Csp1 and VAMP2 was tested by Western blotting. [^{35}S]Csps, [^{35}S]VAMP, and [^{35}S]-SNAP-23 were synthesized by coupled transcription and translation using the TNT *in vitro* system (Promega). Free

[^{35}S]cysteine or [^{35}S]methionine was removed by using Micro-10 columns (Amicon Inc., Beverly, MA).

Dialyzed GST or GST fusion proteins (10 μg) were coupled to 30 μL of glutathione−Sepharose beads (1:1 suspension in buffer A) and incubated overnight with 1×10^6 dpm of ^{35}S-labeled probes in buffer A containing 0.25% BSA at 4 °C. The total reaction volume was 400 μL. Subsequently, supernatants were separated by centrifugation for 5 min at 4 °C (20000*g*), and the beads were washed twice with 1 mL of intracellular buffer containing 0.5% BSA and once with intracellular buffer without BSA. Beads were resuspended in 80 μL of SDS−PAGE sample buffer, boiled for 3 min, and briefly centrifugated. The resulting supernatant (8 μL) was applied for scintillation counting (duplicates), and 60 μL was separated on a 12% SDS−PAGE gel followed by autoradiography of the dried gels.

Cross-Linking. Cells were detached with Krebs Ringer buffer (KRB) [125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 2 mM $MgSO_4$, 25 mM Hepes, 0.4 mM EGTA, 5 mM glucose, and 0.1% BSA (pH 7.4)] containing 10 mM EDTA at 37 °C, centrifuged (for 5 min at 2000*g* and 4 °C), resuspended in LC buffer, and permeabilized by the addition of streptolysin O for 5 min at 37 °C. Permeabilization was controlled by Trypan Blue uptake. The permeabilized cell suspension was divided into aliquots, centrifuged, and resuspended with the corresponding intracellular buffers described above except that KCl instead of potassium glutamate was used. Bismaleimidohexane (BMH, Pierce) and disuccinimidyl suberate (DSS, Pierce) were prepared in DMSO at a concentration of 20 mM. BS3 [bis(sulfosuccinimidyl) suberate, Pierce], the water soluble analogue of DSS, was prepared in water. Cross-linking was initiated by the addition of 1 mM (final concentration) cross-linker, or a corresponding amount of DMSO (670 μM), for 15 min at 37 °C. Cross-linking was stopped by the further addition of 0.1 mM glycine (final concentration) at pH 8 for 15 min at 4 °C. Proteins were subsequently solubilized using 1% Triton X-100 for 15 min at 4 °C followed by sonication (three 10 s pulses). Samples were centrifuged at 15000*g* (20 min at 4 °C) and supernatants diluted in SDS−PAGE sample buffer prior to electrophoresis and immunoblotting. For immunoprecipitations from cells transfected with plasmids encoding 2mycCsp1 or Csp1, supernatants were incubated overnight at 4 °C with a monoclonal anti-myc antibody after Triton X-100 extraction and centrifugation. Samples were subsequently incubated with protein G−Sepharose beads for 2 h at 4 °C, and the beads were washed four times with 600 μL of LC buffer prior to being resuspended in 60 μL of SDS−PAGE sample buffer and further analyzed by electrophoresis and immunoblotting.

Signals were imaged and quantified (FluorChem version 2.00, Alpha Innotech Corp.), and the linearity of the signal was tested for each membrane using different exposure times. The amount of dimerized Csp was normalized against the amount of Csp in the absence of a cross-linker, but in the presence of DMSO, which corresponds to the total initial amount of Csp present in the sample.

RESULTS

The Extreme N-Terminus of Csp Does Not Alter the Total Amount of Insulin Release. The extreme N-terminus varies considerably among the different Csp isoforms known or deduced in humans and mice. We first determined the subcellular distribution of full-length and N-terminally truncated Csps by transient expression in hamster insulinoma HIT-T15 cells. The constructs that were used were tagged with an N-terminal myc epitope to distinguish the proteins overexpressed in a subpopulation of cells from endogenous proteins present in all cells (Figure 1). Confocal immunocytochemistry demonstrates that both Csp1 and Csp2 as well as their N-terminally truncated forms ($Csp1_{14-198}$ and $Csp2_{14-167}$, respectively) colocalize to a large extent with insulin as a marker of LDCVs. Detailed statistical image analysis revealed that 89−92% of pixels from insulin and myc signals of different Csp constructs colocalize, except for mycCsp2 for which only 78% coincided ($n = 9-14$; $2p < 0.05$). However, this difference was no longer apparent when taking into account either pixel intensities in colocalization or correlations between channels, the latter ranging from 0.75 ± 0.09 to 0.85 ± 0.03 for the different constructs. The data therefore indicate that the N-terminus is not required for correct targeting.

Prior to examination of the effects on hormone exocytosis, protein expression levels were compared by Western blotting of homogenates from cotransfected cells using either an anti-Csp1 antibody or an anti-myc antibody (Figure 2A). The anti-Csp1 antibody revealed a band of approximately 35 kDa in transfected as well as control cells indicating the presence of endogenous Csp1. Cotransfection with myc-tagged Csp1 or $Csp1_{14-198}$ constructs resulted in the appearance of a second band with a slightly higher molecular weight, as would be expected for the myc-tagged proteins, and of comparable intensities as detected by the polyclonal anti-Csp1 antibody. Since the antibody is specific for Csp1, only endogenous Csp1 was visualized after coexpression of Csp2 constructs. The anti-myc antibody did not reveal any staining in the controls, but stained corresponding bands in homogenates from transiently transfected cells. Interestingly, both myc-tagged N-terminally truncated Csps stained to a lesser degree than the full-length protein using the anti-myc antibody, whereas the anti-Csp1 antibody recognized myc-Csp1 and myc-$Csp1_{14-198}$ to the same extent. This indicates a possible influence of the adjacent amino acids in the recognition of the myc epitope. To verify this observation, we have repeated the experiments using in addition a distinct commercial anti-Csp antibody (A1567) directed against the soluble domains of the protein (Figure 2B). Endogenous Csp, myc-tagged Csp1, and myc-tagged $Csp1_{14-198}$ migrate at different distances, excluding the possibility that overexpression of endogenous Csp1 may have taken place. Both anti-Csp antibodies recognized myc-Csp1 and myc-$Csp1_{14-198}$ to the same extent. In contrast, only a faint signal was detected by the anti-myc antibody in the case of myc-$Csp1_{14-198}$. Note also that in cells overexpressing myc-Csps the monoclonal anti-myc antibody recognizes a faint band migrating slightly faster than the major band and comigrating with endogenous Csp1. This signal corresponds to nonacylated myc-Csp1 and slightly enhances the minor signal in blots incubated with the polyclonal antibodies. A similar observation concerning the reactivity of a myc epitope has been reported for another membrane protein (*29*). It is therefore reasonable to assume that Csp2 and $Csp2_{14-167}$ were expressed at comparable levels. Like immunoblots using the anti-Csp1 antibody, an

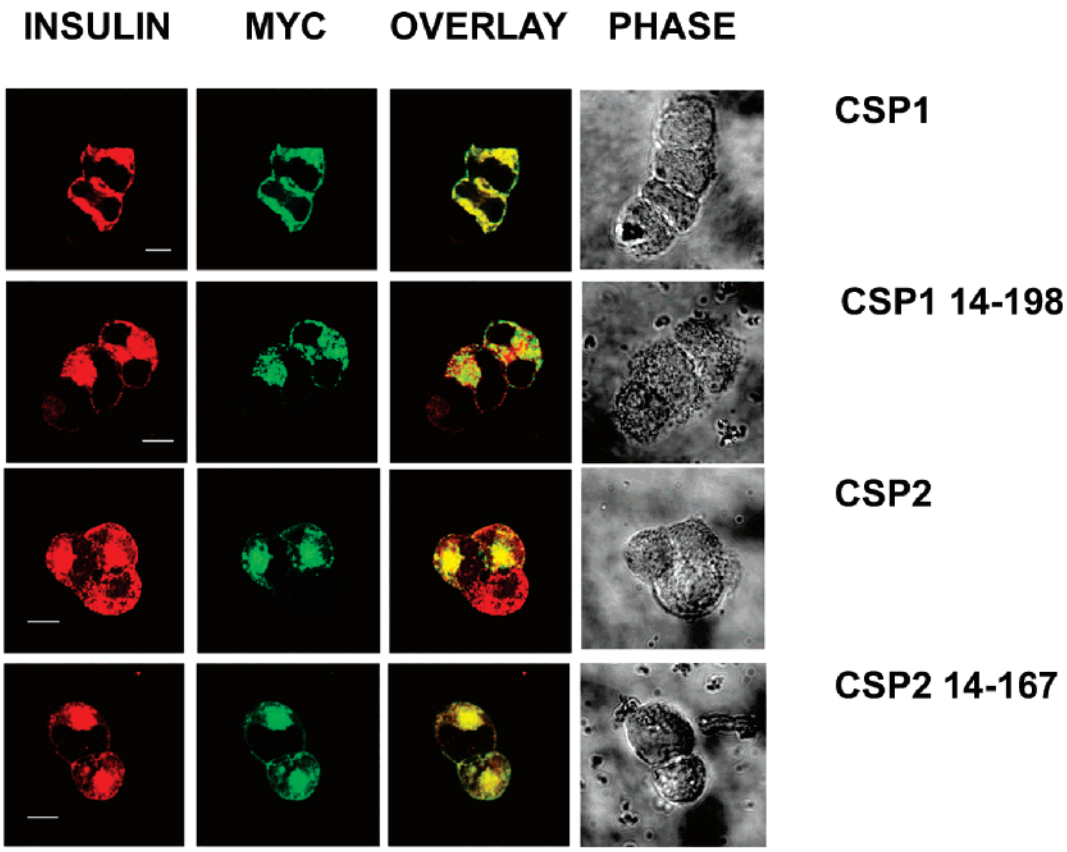

FIGURE 1: Localization of wild-type and N-terminally truncated Csps upon expression in insulin-secreting HIT-T15 cells. Cells were seeded on glass coverslips and transiently cotransfected with the indicated constructs. Two days later, cells were double-stained with a polyclonal anti-insulin antibody (insulin) and a monoclonal anti-myc antibody (myc). Insulin staining was detected with a rhodamine-conjugated anti-guinea pig IgG, and immunostaining of myc-Csps was revealed by fluorescein-coupled anti-mouse IgG.

additional band of 33 or 27 kDa appeared for Csp1 or Csp2, respectively. These bands represent most likely a minor amount of nonpalmitoylated Csps.

To test for a putative function in exocytosis, we resorted to a well-established transient cotransfection system which employs a plasmid encoding human preproinsulin (phINS) as a reporter gene. The release of the resulting insulin split product, human insulin C-peptide, reliably reflects peptide hormone release from cotransfected cells only. Cotransfected HIT-T15 cells were exposed to Krebs Ringer buffer alone or stimulated by 50 mM KCl (Figure 2 C), which causes a depolarization-induced Ca^{2+} influx through voltage-dependent Ca^{2+} channels. As reported previously, overexpression of full-length Csp1 or Csp2 results in a profound inhibition of stimulated hormone release as compared to control cotransfections using pcDNA3. This effect is most likely due to scavenging of other proteins required for exocytosis. Note that only stimulated, but not basal, levels of insulin C-peptide release were altered, which is in line with the proposed specific role of Csps during stimulated exocytosis only. Transient expression of $Csp1_{14-198}$ and $Csp2_{14-167}$ inhibited the release of human C-peptide to the same extent as full-length constructs. Therefore, the first 13 amino acids at the N-terminus of Csps are not important for membrane association or for overall insulin release as measured by biochemical methods.

Effect of C- and N-Terminal Truncations of Csp on Localization and Exocytosis. Csp binds a number of proteins relevant to exocytosis or its regulation, and the corresponding domains have been partially mapped. As the precise residues involved in interaction were in most cases unknown, we used truncated forms of Csp to evaluate their effect in insulin exocytosis. We employed constructs (see Figure 3) which encompass either full-length Csp, only the J-domain and the variable N-terminus (Csp_{1-82}), the N-terminal part including the linker region (Csp_{1-110}), or the C-terminal half of the protein ($Csp1_{83-198}$). All these constructs were tagged with an N-terminal myc epitope to allow selective detection in transfected cells.

Expression of only the N-terminus and the J-domain common to all mammalian Csps (Csp_{1-82}, Figure 3) in HIT-T15 cells resulted in uniform staining of the cell, except for the nucleus, which is indicative of a cytosolic distribution. In contrast, expression of Csp containing the J-domain including the linker region (Csp_{1-110}) partially colocalized with insulin-containing secretory granules, and this was not altered by the E93V mutation, a position important for Csp action (*13*). Expression of the C-terminal half of Csp1 ($Csp1_{83-198}$, Figure 3) or of Csp2 ($Csp2_{83-167}$, data not shown) resulted in a granular pattern, but colocalization was observed to a lesser extent than for wild-type Csp. The levels of expression were compared in immunoblots using Tricine gels to resolve the truncated peptides with low molecular weights (Figure 4, top panel).The anti-myc antibody exhibits nonspecific reactions with 43 and 29 kDa bands in homogenates from nontransfected cells (Figure 4, top panel, CON). However, truncated peptides migrated at the expected molecular weights but were expressed to a lower extent than

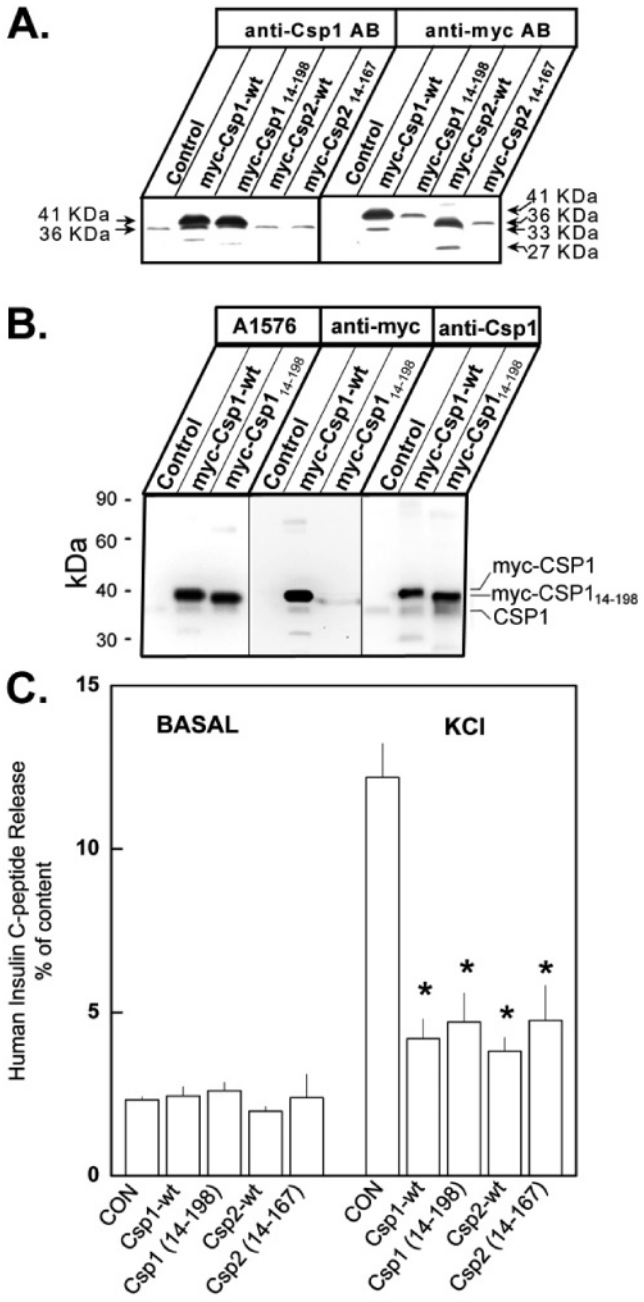

FIGURE 2: Expression and regulation of insulin secretion by full-length Csps and N-terminal deletion mutants. HIT-T15 cells were transiently cotransfected with plasmids encoding human preproinsulin and with pcDNA3 vector (control, CON) or with pcDNA3 encoding Csps or indicated N-terminal truncations of Csp1 or Csp2. Two days later, cells were analyzed. (A and B) Immunoblot analysis using 40 μg of proteins/lane separated on a 12% mini-SDS−PAGE gel, transferred to PVDF membranes, and incubated with the indicated antibodies. (C) Cells were washed and kept in Krebs Ringer buffer (KRB) alone (basal) or stimulated by the addition of 50 mM KCl (KCl). Human insulin C-peptide release, reporting secretion from cotransfected cells, was assessed with an ELISA. $N = 6-10$ from at least three independent experiments. An asterisk means $2p < 0.05$ as compared to control.

full-length Csps. Comparable amounts were detected by anti-myc antibodies for Csp_{83-198} (20 kDa), Csp_{1-110} (15 kDa), Csp_{1-110}E93V (15 kDa), Csp_{83-198}, Csp_{1-110}, and Csp_{1-110}-E93V, whereas only minor amounts of Csp_{1-82} (14 kDa) were visualized. Anti-Csp1 antibodies detected equal amounts of Csp_{1-110}, Csp_{1-110}E93V, and Csp_{1-82}, indicating again that detection of the myc epitope is problably context-dependent. Csp_{83-198} was not detected by anti-Csp, consistent with the observation that the J-domain is part of the epitope. Assessment of insulin secretion from cotransfected cells revealed that $Csp1_{83-198}$ and Csp_{1-110} inhibited hormone release, although to a smaller extent than the full-length protein (Figure 4, bottom panel). Like previous results, the E93V mutation in the linker region abolished the effect of Csp_{1-110}. In contrast, the J-domain alone (Csp_{1-82}) did not alter insulin secretion. $Csp1_{137-198}$ was only expressed at very low levels as assessed by immunofluorescence and immunoblots, and did not change insulin release (data not shown).

Isoform and Calcium-Dependent Interaction of Csp with VAMP. In our attempt to identify proteins interacting with Csp in our model system, immunoprecipitations were performed using extracts from HIT-T15 cells. As initial experiments conducted in the presence of EGTA (without calcium added) were negative, we examined the possible effect of calcium-induced exocytosis on protein−protein interactions. As shown in Figure 5, immunoprecipitation of endogenous Csp1 under stimulating conditions (10 μM free calcium) led to the co-immunoprecipitation of the SNARE protein VAMP2 (11.9 ± 4.5% of the total VAMP2). Immunoprecipitation using an antibody directed against VAMP2 also coprecipitated Csp1 (9.7 ± 3.6% of the total Csp1), and again, the effect was only observed in the presence of 10 μM free calcium. In contrast, the SNARE protein syntaxin 1 or the marker protein for synaptic-like microvesicles, synaptophysin, was not coprecipitated with either low (0.1 μM) or high (10 μM) calcium.

To test whether the observed interaction may be direct or indirect, we performed binding assays using recombinant proteins and radioactive, in vitro-translated products (Figure 6). ^{35}S-labeled Csp1 bound significantly to GST−VAMP2 and to the cytosolic domain of Ti-VAMP/VAMP7, but not to VAMP3/cellubrevin in a buffer containing residual Ca^{2+} levels (no EGTA added, Figure 6A). Note that Ti-VAMP is expressed in primary islet cells and the derived clonal rat, hamster, and mouse cell lines INS-1E, HIT-T15, and MIN6, respectively (data not shown). We also assessed the binding of another SNARE protein, SNAP-23, known to bind to the three recombinant VAMP proteins that were used. [^{35}S]-SNAP-23 bound, as expected, to all three VAMP proteins, suggesting that the absence of binding of [^{35}S]Csp1 to GST−VAMP3 is not due to a major alteration of the recombinant protein (Figure 6B). Next we examined whether any difference in binding can be observed between the two Csp splice variants, Csp1 and Csp2, and whether the point mutation in the linker region (E93V) has any influence. This point mutation considerably alleviates the effect of Csp overexpression on insulin secretion (see above and Figure 4). Whereas [^{35}S]Csp1 and [^{35}S]Csp1E93V bound GST−VAMP2, [^{35}S]Csp2 did not bind to a significant extent (Figure 6C). Finally, we performed binding experiments using recombinant GST−Csp and in vitro-translated [^{35}S]-VAMP (Figure 6D). Again, binding was observed only between Csp1 and VAMP2, but not in the case of Csp2.

Calcium-Dependent Dimerization of Csp. The conditions employed in immunoprecipitation favor the detection of stable complexes, although lower-affinity interactions may be physiologically relevant. We therefore resorted to chemical cross-linking of Csp in streptolysin O-permeabilized HIT-

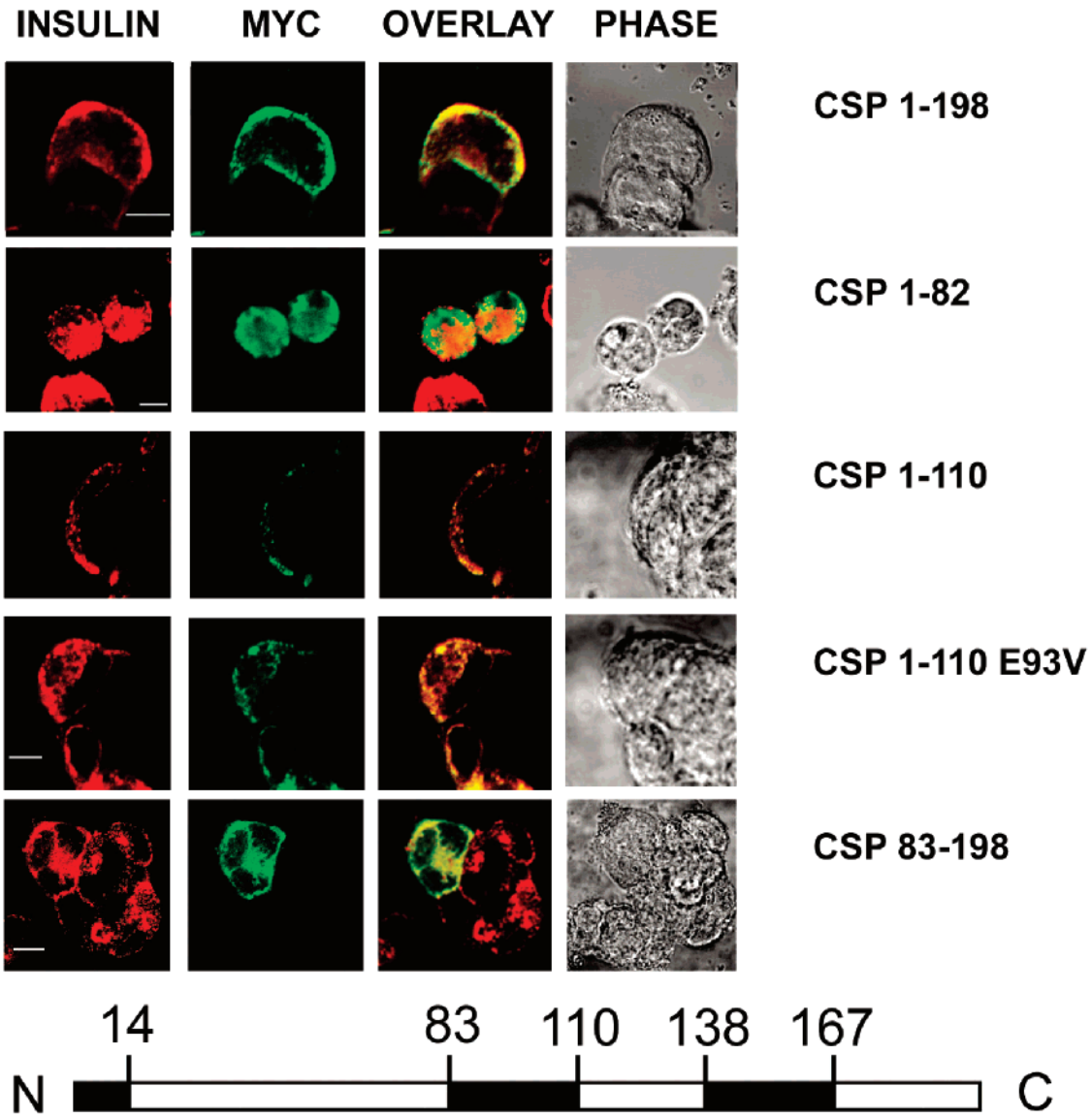

FIGURE 3: Localization of wild-type and C-terminally truncated Csps upon expression in insulin-secreting HIT-T15 cells. Cells were handled as described in the legend of Figure 1. Insulin staining was detected with a rhodamine-conjugated anti-guinea pig IgG, and immunostaining of myc-Csps was revealed by using fluorescein-coupled anti-mouse IgG.

15 cells under basal (0.1 μM) and stimulatory concentrations (10 μM) of free calcium as imposed by calcium/EGTA buffers. The cross-linker used, disuccinimidyl suberate, reacts with the ϵ-amine of lysine. The experimental conditions have been selected to prevent complete cross-linking and favoring the appearance of one or few additional species within the separating range of the gel. Under native conditions, an additional band of 45 kDa was observed; this band was neither altered by calcium (Figure 7A) nor detected using a commercial anti-Csp antibody (data not shown). Under cross-linking conditions, an additional band of 62 kDa was detected with an anti-Csp antibody (Figure 7A). The intensity of this 62 kDa band increased in the presence of stimulatory calcium levels by approximately 50% ($2p < 0.05$; see Figure 7A,D). Note that all conditions included equal percentages of DMSO and that the carrier DMSO alone did not alter migration or induce additional bands (data not shown). We also tested other reagents, such as BS3, a water-soluble analogue of disuccinimidyl suberate, and bismaleimidohexane which cross-links cysteines. In both cases, we could not detect any additional band within the separation range of the gel. Note that most cysteines are probably acylated in Csp (*14*).

Using antibodies directed against VAMP2 or syntaxin, we could not detect any immunoreactivity at 62 kDa, suggesting that these proteins are not part of the complex (data not shown). The observed molecular mass of the 62 kDa band is, however, compatible with the approximate size of a Csp dimer, taking into account that cross-linked proteins often migrate at a molecular mass slightly lower than the predicted value. To test this hypothesis, we took advantage of the observation that Csp1 constructs tagged with a 2myc epitope exhibit a considerable shift in migration during SDS−PAGE (Figure 7B). Western blot analysis using an anti-Csp antibody of cells expressing 2myc-Csp1 revealed two major bands of 34 and 38 kDa representing endogenous Csp1 and 2myc-Csp1, respectively. After cross-linking by DSS, an additional band at approximately 80 kDa is apparent. Transiently expressed Csp1 migrates at a molecular mass of 34 kDa and was not separated from endogenous Csp1, and cross-linking resulted in an additional band at 62 kDa (Figure 7B).

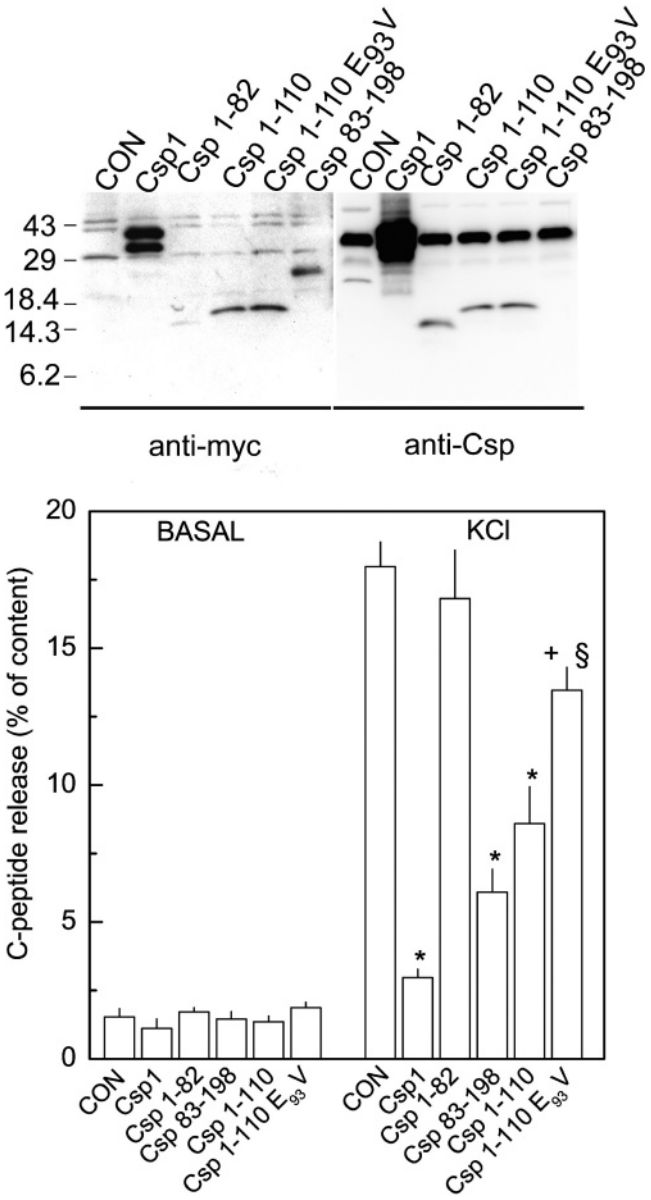

FIGURE 4: Regulation of insulin secretion by Csp truncation mutants. In the top panel are shown expression levels of Csp constructs. Homogenates of cells transiently cotransfected with plasmids encoding the indicated myc-tagged constructs were separated on Tricine gels, subsequently transferred to PVDF membranes, and stained with anti-myc or anti-csp antibodies. Migration of molecular mass markers is indicated in kilodaltons. In the bottom panel, cells were handled as described in the legend of Figure 2 and either kept in Krebs Ringer buffer (KRB) alone (basal) or stimulated by the addition of 50 mM KCl (KCl). Human insulin C-peptide release, reporting secretion from cotransfected cells, was assessed with an ELISA. $N = 6-13$ from at least three independent experiments. An asterisk means $2p < 0.05$ as compared to control. A plus sign means the value is not significantly different from control. The section mark means $2p < 0.05$ as compared to Csp_{1-110}.

Transient cotransfection of cells with plasmids encoding both proteins, 2myc-Csp1 and Csp1, resulted in an increase in the magnitude of the 34 kDa band (as compared to that for 2myc-Csp1) and the 38 kDa band of 2myc-Csp1. Cross-linking in these cells (Figure 7B, right lanes) produced a 62, 70, and 80 kDa band, which is compatible with the presence of Csp1 dimers at 62 kDa, Csp1−2myc-Csp1 dimers at 70 kDa, and 2myc-Csp1 dimers at 80 kDa. This interpretation was further supported by immunoprecipitations using an anti-myc antibody (Figure 7B, right lanes) as the 80 kDa band was precipitated from cells overexpressing 2myc-Csp or 2myc-Csp and Csp, whereas the70 kDa band was only precipitated from cells overexpressing Csp.

Both Csp1 and Csp2 are localized to insulin-containing LDCVs upon their overexpression, and differ only in their C-terminus. We were therefore interested in whether both isoforms can be cross-linked and whether cross-linking depends on calcium levels (Figure 7C). Although mRNA encoding Csp2 is present in insulin-secreting cells (*13*), the protein cannot be detected by our antibody. For this reason, we resorted to transient expression of epitope-tagged proteins. Transiently expressed Flag−Csp1 can indeed be cross-linked in a calcium-sensitive manner similar to that for endogenous Csp1 (Figure 7D). This was also observed for Flag−Csp2, though the pronounced diminution of the non-cross-linked band suggests that additional complexes with high molecular masses are generated but may not have entered the gels. The point mutant Csp2E93V was cross-linked to an extent similar to that of wild-type Csp2 (data not shown). Most interestingly, the influence of stimulatory calcium levels was more pronounced in the case of Flag−Csp2 than in the case of Flag−Csp1 (Figure 7D). Similar results were obtained using the myc epitope, and again, the observed extent of cross-linking in the case of Csp2 exceeded that of Csp1 at 0.1 and 1 μM Ca^{2+} by 69 ± 4 and 164 ± 73%, respectively, in three independent experiments ($2p < 0.05$ as compared to Csp1). The different propensity for Csp1 or Csp2 to undergo cross-linking could not be explained by differences in expression levels as this effect was still observed after variation of expression levels by a factor of 3 (data not shown). Note also that both transiently expressed proteins contain the same number of lysines available for the cross-linker. Our data therefore suggest that the C-terminus of Csp influences its homodimerization.

DISCUSSION

In this study, we examined the functional domains of Csp in the exocytosis of LDCVs and provide evidence for a crucial role of the C-terminus in its regulation and the protein's capacity to form protein−protein interactions. Indeed, a number of such interactions have been described previously, such as with chaperone proteins (*11*, *13*, *20*, *30*), the SNARE proteins VAMP2 (*16*) and syntaxin 1 (*22*, *24*), neuronal calcium channels (*16*) and associated G-proteins (*18*, *19*), and the cystic fibrosis transmembrane conductance regulator (*31*). However, in most cases, synaptic or transport vesicles have been studied, whereas little is known as far as LDCVs and non-neuronal systems are concerned.

Sequence comparison of known or predicted Csps reveals not only conserved domains but also two regions of striking diversity: the N- and C-termini. Clearly, the N-terminus is not required for targeting of Csp to vesicles and did not contribute to the overall inhibition observed upon transient expression of the wild-type form. This observation is in line with results obtained in chromaffin cells (*32*), where mutation of the conserved phosphorylation site within the N-terminus mainly changed release kinetics, but not the total amount released. Note that the identified binding protein, synaptotagmin I, is expressed and functional in the cell lines that

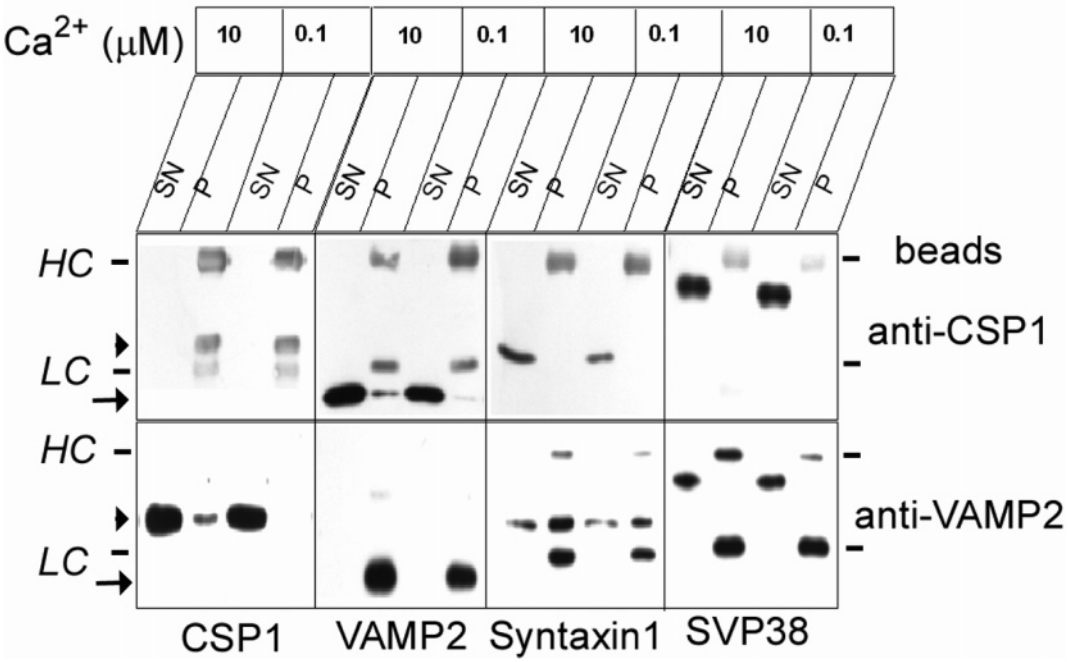

FIGURE 5: Ca^{2+}-dependent co-immunoprecipitation of Csp1 and VAMP2. Crude HIT-T15 membranes (200 μg) were extracted in the presence of 0.1 μM Ca^{2+} or 10 μM Ca^{2+} for 2 h at 4 °C. After centrifugation, supernatants were incubated overnight with anti-Csp1 or anti-VAMP2 antibodies coupled to Sepharose beads (beads anti-Csp1, anti-VAMP2), and subsequently, beads were washed as described in Experimental Procedures. Aliquots from supernatant (SN) and pellet (P) fractions were applied to a 12% SDS−PAGE gel and immunoblotted using polyclonal anti-Csp1 antibodies (Csp1) or monoclonal anti-VAMP2 (VAMP2), anti-syntaxin 1 (syntaxin 1), or anti-synaptophysin (SVP-38) antibodies. Lines indicate positions of the IgG heavy chain (HC, 55 kDa) and light chain (LC, 25 kDa); the positions of Csp1 (arrowheads) and VAMP2 (arrows) are also given.

were studied (*33*, *34*). Such kinetic alterations cannot be detected by our biochemical release assay.

Functional data and interaction studies indicate that the different domains of Csp interact with distinct proteins and their concerted action leads to the physiological effect (*13*, *20*). The J-domain (Csp_{1-83}) has been shown to be sufficient for binding to and activation of the chaperone Hsc70 (*11*−*13*), and this is not changed by the E93V mutation in the Csp linker region (*13*). We cannot draw conclusions from the transient overexpression of Csp_{1-83} alone as expression was cytosolic and relevant levels may not be attained at membranes. However, both Csp_{1-110} and the mutant Csp_{1-110}E93V can interact with Hsc70 and stimulate its ATPase activity, but only Csp_{1-110} altered insulin secretion. We can obviously not exclude the possibility that functional changes after overexpression as used here may reflect sequestering of components of the secretory machinery. Nevertheless, our current observation is in line with our previous findings that interfering with Csp−Hsc70 interactions is not sufficient for production of the profound inhibition of LDCV exocytosis as seen on Csp overexpression (*13*). It has been shown recently that separate deletions either in the linker region or in the C-terminus only marginally impair Csp function in *Drosophila* (*35*). This observation is compatible with our findings as mutations in the linker affect peptide hormone secretion only in the absence of the extended C-terminus (this study and ref *13*).

To our surprise, Csp_{1-110} was still localized to membranes, although it lacked the cysteine string whose palmitoylation is required for initial vesicle attachment, but not for stable membrane association (*24*, *36*). Our data do not contradict these observations since protein interactions of the linker, as suggested by functional data (*13*), or the mainly hydrophobic nature of amino acids 98−110 may lead to a secondary membrane attachment, thus providing an artificial though useful probe.

In neurons, Csp has been shown to form trimeric complexes containing chaperones such as Hsc70 (*30*) or Hsp90 (*17*). Hsp90 and Csp are complexed with αGDP dissociation inhibitor to form a Rab recycling complex (*17*). Although the precise binding sites of Hsp90 on Csp have not been mapped, it is reasonable to assume that the J-domain is concerned. Hsc70 forms trimeric complexes with Csp and the ubiquitous cochaperone αSGT (small glutamine-rich tetratricopeptide repeat-containing protein) on synaptic vesicles (*20*). Overexpression of SGT inhibits neuroexocytosis and is largely absent from synaptic vesicles in Csp knockout mice, indicating a physiological role for this trimer (*20*). The site for SGT binding to Csp has been mapped to the cysteine string motif (amino acids 117−140) (*21*). Although interactions with SGT may contribute to the observed effects in our system with regard to the effect of full-length Csp, the current study clearly indicates that additional interactions have to be invoked in LDCV exocytosis. Indeed, overexpression of constructs excluding the domain binding for αSGT, such as Csp_{1-110}, interfered with insulin secretion, although to a lesser extent than full-length Csp1.

The J-domain (Csp_{1-83}) also interacts with the α-subunit of heterotrimeric G-proteins, G_α, and thereby inhibits N-type Ca^{2+} channels (*18*, *19*). Moreover, a direct interaction with P/Q-type Ca^{2+} channels has been reported for Csp1, though the binding site within Csp is unknown (*16*). The absence of an effect of the J-domain in our study, as found for Csp_{1-110}E93V, on depolarization-induced insulin secretion

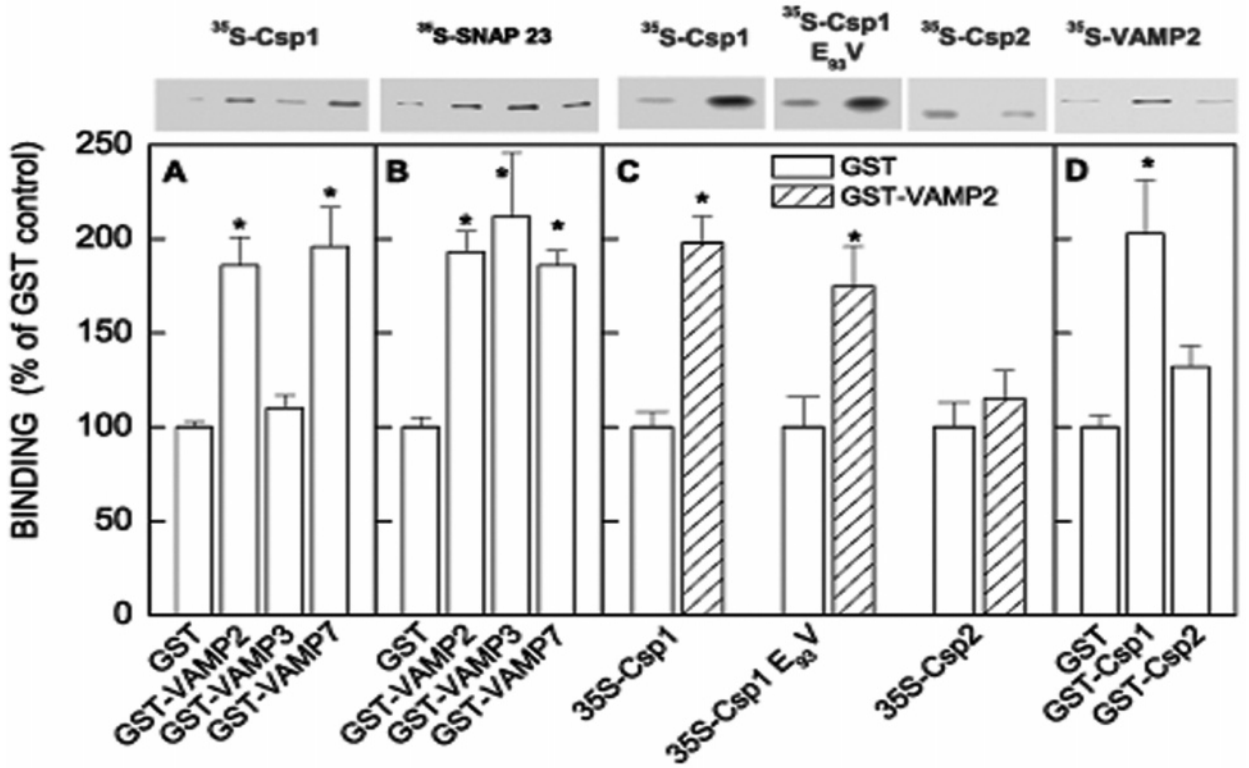

FIGURE 6: Csp1, but not Csp2, binds to VAMP2 and VAMP7 in vitro. Across the top are autoradiography results for bound probes separated by SDS−PAGE. (A) Purified GST, GST−VAMP2, GST−VAMP3, or GST−VAMP7 (10 μg) was incubated with 30 μL of pre-equilibrated glutathione−Sepharose beads in the presence of 10^6 dpm of [^{35}S]Csp1 in calcium-containing buffer A with BSA (0.25% BSA) at 4 °C overnight. Bound [^{35}S]Csp1 was quantified by scintillation counting and verified by autoradiography. $N = 3-5$. (B) Same as panel A, but beads were incubated with 10^6 dpm of [^{35}S]SNAP-23. $N = 3$. (C) GST or GST−VAMP2 (10 μg) was incubated as described for panel A using 10^6 dpm of [^{35}S]Csp1, [^{35}S]Csp1E93V, or [^{35}S]Csp2. $N = 3$. (D) GST, GST−Csp1, or GST−Csp2 (10 μg) was incubated with 10^6 dpm of [^{35}S]VAMP2 and analyzed as described above. $N = 3$. An asterisk means $2p < 0.05$.

is most likely due to the use of different systems. L-Type but not N- or P/Q-type Ca^{2+} channels play the major role in insulin secretion (*37*). Moreover, Csp overexpression does not affect voltage-dependent Ca^{2+} channel activity in clonal β-cells (*10*) and exerts comparable effects in intact and permeabilized cells (*9*). Indeed, care must be taken in comparing results between different cellular systems. Interactions with syntaxin have not been found in all systems, and overexpression of Csp induces distinct phenotypes in different preparations. Whereas no effect was observed during exocytosis at *Drosophila larvae* neuromuscular junctions (*22*), exocytosis of LDCVs is largely inhibited in chromaffin and in insulin-secreting cells (*10*, *13*, *38*). This may reflect distinct binding partners or different stoichiometries.

We observed two interactions of Csp which are modulated by the C-terminus: interaction with the SNARE protein VAMP2 and homodimerization. We were unable to co-immunoprecipitate the SNARE protein syntaxin 1, which is required for insulin exocytosis (*39*). Although genetic and functional data support such an interaction in *Drosophila* neurons (*22*) and for plasma membrane-bound Csp in adipocytes (*24*), it was not found in mammalian brain extracts (*16*, *23*). One possible explanation for this discrepancy may reside in the physical distance between the binding partners. Whereas tight coupling of vesicles to membranes may be expected in neuronal systems or in the case of the location of both partners in the plasma membrane as in adipocytes, only 7% of LDCVs reside next to the plasma membrane in insulin-secreting cells (*40*). It should also be noted that DnaJ binds peptides with low affinity and detergents may interfere (*41*). In the absence of detectable amounts of syntaxin and synaptophysin, which both may bind to VAMP2 (*42*), we assume that Csp1 interacts with monomeric VAMP2. A considerable amount of plasma membrane-bound SNAREs are indeed present in a monomeric conformation prior to docking or fusion, and the same may apply to the vesicular SNAREs (*43*). As monomeric VAMP tends to be less structured (*44*), it may benefit from the action of a chaperone such as Csp. The calcium dependency of Csp−Csp and Csp−VAMP2 interactions in our cellular system suggests that both interactions are occurring during or after stimulation of exocytosis, as there is no indication for binding of calcium to Csp. These findings also provide some explanation for the observation that the different C-termini are important in exocytosis, and their difference becomes apparent in the presence of additional mutations. Our data do not indicate at what stage of the exo-endocytotic cycle Csp and VAMP interact, but functional data from chromaffin cells and neuroxocytosis largely favor a function for Csp during the exocytotic part of the cycle (*38*, *44*). Cross-linking did not reveal any complex with VAMP. This may be due to the hydrophobic nature of the cross-linker used and the fact that none of the 14 lysines present in Csp1 or Csp2 is found in the variant C-terminus, which is at least required in vitro for VAMP binding.

As Csp1, Csp1E93V, and Csp2 can be cross-linked in permeabilized cells, we assume that neither the linker nor the variant C-terminus is required for homodimerization

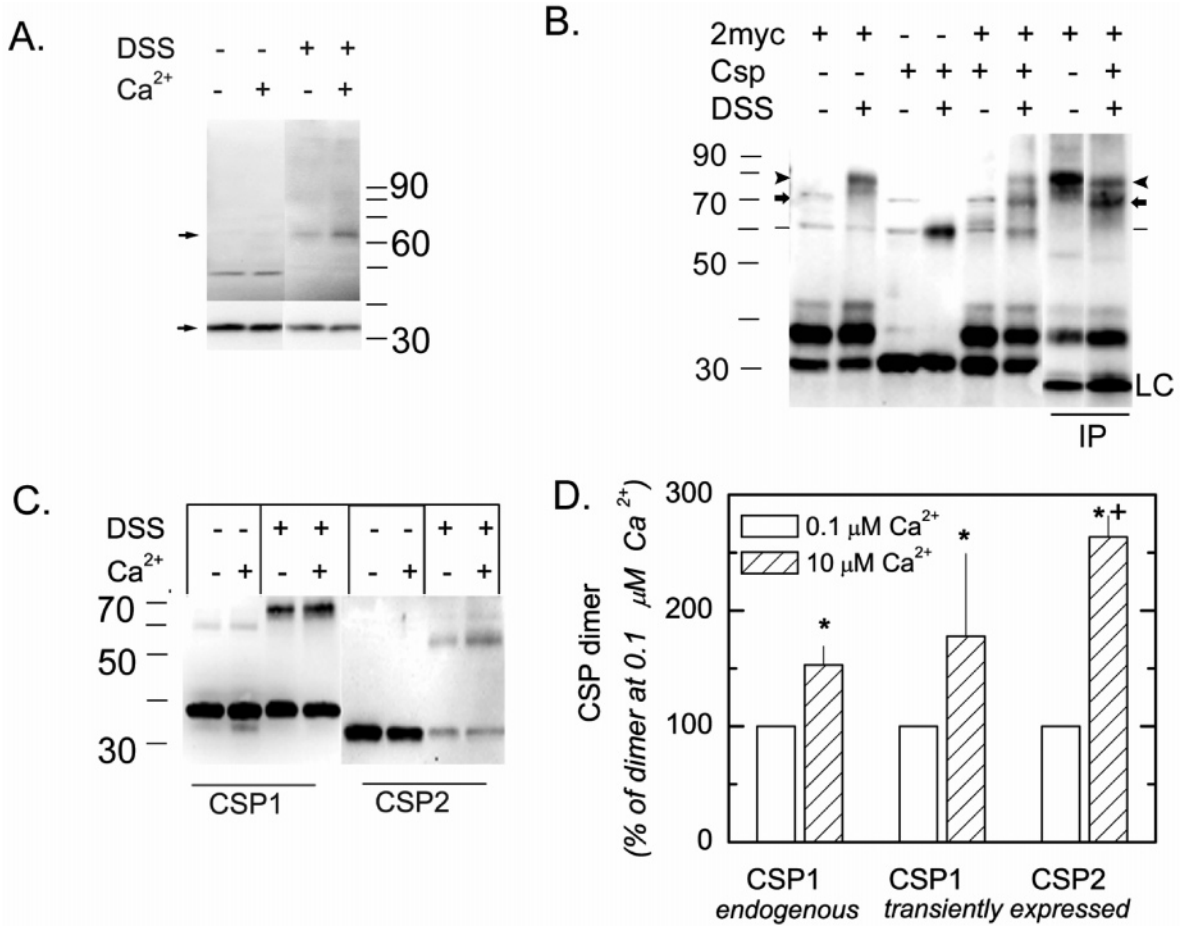

FIGURE 7: Calcium-induced dimerization of Csp1 and Csp2 in permeabilized cells. Streptolysin O-permeabilized HIT-T15 cells were exposed to 0.1 (−) or 10 μM (+) free calcium in the absence (−) or presence (+) of a cross-linking reagent (disuccinimidyl suberate, DSS). DMSO was present under both conditions. Subsequently, proteins were solubilized and subjected to SDS−PAGE followed by immunoblots. Molecular mass standards are given at 10 kDa intervals. (A) Calcium-dependent cross-linking of endogenous Csp1 as revealed by immunoblots using an anti-Csp antibody. Monomeric and dimeric forms are indicated with arrows. (B) Cross-linking of Csp1 in cells transiently expressing either 2myc−Csp1 (2myc), Csp1 (wt), or both constructs revealed by immunoblots using an anti-Csp antibody. Cross-linked transiently expressed proteins are indicated with different symbols (arrowheads for 2myc−Csp1/2myc−Csp1, lines for 2myc−Csp1/Csp1, and arrows for Csp1/Csp1). Analysis was performed either on cell homogenates or after immunoprecipitation with an anti-myc antibody (IP). LC represents the immunoglobulin light chain. (C) Calcium-dependent dimerization of Flag−Csp1 and Flag−Csp2 revealed by anti-Flag antibodies. (D) Calcium-dependent dimerization of Csp1 and Csp2. Amounts of dimers were normalized over Csp expression in native samples (for details, see Experimental Procedures). $N = 3-6$. An asterisk means $2p < 0.05$ vs 0.1 μM Ca^{2+}. In the *t*-test, a plus sign means $p < 0.05$ vs Csp1 (one-tailed ANOVA).

itself. In vitro studies using recombinant proteins have mapped dimerization sites to the linker and cysteine string (*45*), a region containing a large stretch of hydrophobic amino acids. Under our conditions in permeabilized cells, these residues are most likely buried in the membrane bilayer. It is noteworthy that this hydrophobic region contains three of the 14 available lysines. The lack of efficiency of the water-soluble DSS analogue may indicate that these residues are in the vicinity. The role of hydrophobic domains has also been demonstrated for the dimerization of other DnaJ domain-containing proteins, namely, Sis1 and DjlA, and seems to be required for their chaperone activity, but not for the activation of heat-shock proteins. Note also that a Csp dimer is probably present in the reconstituted Csp−Hsc70−SGT complex (*20*). Dimerization in insulin-secreting cells was stimulated by levels of Ca^{2+} that induce exocytosis and interactions between Csp1 and VAMP2. It is tempting to speculate that these events occur simultaneously. The interaction between the terminus of Csp1 and VAMP may relieve some constraint on Csp homodimerization. In contrast, Csp2, not being restricted by the variant C-terminus, can form a dimer more often.

In conclusion, we have demonstrated that the linker region and the C-terminus of Csp are interfering with exocytosis and could assign two functions to the C-terminus in Ca^{2+}-stimulated cells: interaction with the SNARE protein VAMP and modulation of Csp homodimerization. What may be the role of these events? Csp has a chaperone activity of its own, which may be favored by dimerization in analogy to other J-domain-containing chaperones (*46*, *47*). This process may also increase the local concentration of Csp at docking and fusion sites. Moreover, Csp has been implicated in a number of distinct interactions, and it is still unclear which interaction occurs at what stage (*15*, *17*, *20*, *22*). They may be organized either sequentially or by functionally different Csps. Although it remains a hypothesis at present, dimerization could favor simultaneous interactions with distinct partners at the same location.

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