Functional role of J domain of cysteine string protein in Ca\(^{2+}\)-dependent secretion from acinar cells

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Weng N, Baumler MD, Thomas DDH, Falkowski MA, Swayne LA, Braun JEA, Groblewski GE. Functional role of J domain of cysteine string protein in Ca\(^{2+}\)-dependent secretion from acinar cells. Am J Physiol Gastrointest Liver Physiol 296: G1030–G1039, 2009. First published March 12, 2009; doi:10.1152/ajpgi.90592.2008.—The heat shock protein 70 family members Hsc70 and Hsp70 are known to play a protective role against the onset of experimental pancreatitis, yet their molecular function in acini is unclear. Cysteine string protein (CSP-\(\alpha\)) is a zymogen granule (ZG) membrane protein characterized by an NH\(_2\)-terminal “J domain” and a central palmitoylated string of cysteine residues. The J domain functions as a co-chaperone by modulating the activity of Hsc70/Hsp70 family members. A role for CSP-\(\alpha\) in regulating digestive enzyme exocytosis from pancreas was investigated by introducing CSP-\(\alpha\) truncations into isolated acini following their permeabilization with Perfringolysin O. Incubation of basal secretion measured in the presence of inositol triphosphate (IP3) and diacylglycerol, which, in turn, cause the release of Ca\(^{2+}\) from intracellular stores and activation of protein kinase signaling pathways (reviewed in 49). Although multiple signaling pathways are activated by secretagogues, analysis of IP\(_3\) receptor knockout mice have conclusively demonstrated that the acute elevation of cytosolic Ca\(^{2+}\) is an essential messenger to trigger ZG exocytosis and digestive enzyme secretion into the pancreatic duct (19).

The constitutively expressed cognate heat shock protein 70 (Hsc70) and stress-inducible Hsp70 are chaperone proteins that are expressed in acinar cells and, in the case of Hsp70, highly induced in response to various stressors both in isolated acini and in vivo (2, 44). Induction of Hsp70 expression before initiating acute experimental pancreatitis by treatment with high supraphysiological concentrations of cholecystokinin or its analog cerulein was shown to provide a protective role against the onset of acinar damage marking the earliest stages of pancreatitis (2, 44). The earliest events that accompany hyperstimulation of acini are the inhibition of normal apical secretion, basolateral exocytosis of ZGs (15), premature activation of trypsinogen, and formation of large cytoplasmic vacuoles (reviewed in 27). Perhaps not surprisingly given the highly differentiated secretory function of the acinar cell, these aberrant alterations in acinar function occur largely within the secretory pathway and, therefore, infer that the Hsp70-mediated protective effects are somehow targeted to regulatory proteins important for this process.

Cysteine string protein (CSP-\(\alpha\)) is a ZG membrane protein bearing a signature “J domain” and a cysteine-rich string region (5). In addition to its presence on ZGs, CSP-\(\alpha\) is found on synaptic vesicles (30), clathrin-coated vesicles (3), endocrine granules (8, 50), and neuroendocrine granules (9, 26). CSP-\(\alpha\) associates with and stimulates the ATPase of heat shock protein 70 (Hsp70) family members including Hsc70 (6, 39) and Hsp70 (10). Over 40 human proteins contain a J domain and an ~70-amino-acid signature domain that activates the ATPase activity of the Hsc70 family to mediate conformational changes in a number of cellular processes (reviewed in Refs. 36 and 52). Aside from CSP-\(\alpha\), which is one of the better characterized J proteins, clear functional roles have been described for only a few J proteins. They are generally thought to provide specificity to cellular conformational work by targeting client proteins to Hsc70 and thereby targets Hsc70 activity to the secretory pathway in cells, CSP-\(\alpha\) and Hsc70 are reported to undergo dynamic associations with several accessory proteins including small glutamine-rich tetratricopeptide repeat domain protein (43), Hsp70 organizing protein (36), and Hsp70 interacting protein (36), which also likely regulate chaperone activity. The ATPase activity of Hsc70 is coupled to a wide range of cellular folding processes that include folding of newly synthesized proteins, refolding of misfolded proteins, as well as conformational changes of components in signal transduction and apoptotic pathways (reviewed in 7). How different members of the J protein family exploit Hsc70 for

ZYMOCORE N (ZGs) of exocrine pancreatic acinar cells are secretory organelles responsible for the regulated secretion of digestive enzymes. In response to a meal, secretagogues including cholecystokinin and acetylcholine activate G protein-coupled receptor signaling pathways to generate inositol 3,4,5-trisphosphate (IP3) and diacylglycerol, which, in turn, cause the release of Ca\(^{2+}\) from intracellular stores and activation of protein kinase signaling pathways (reviewed in 49). Although multiple signaling pathways are activated by secretagogues, analysis of IP3 receptor knockout mice have conclusively demonstrated that the acute elevation of cytosolic Ca\(^{2+}\) is an essential messenger to trigger ZG exocytosis and digestive enzyme secretion into the pancreatic duct (19).

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distinct conformational work in the cell is a major biological question to be addressed. Identification of the cellular target(s) for the CSP-α/Hsc70 conformational work in neurons and endocrine cells has been controversial. Independent reports have emerged supporting a role for CSP-α in facilitating conformational changes in soluble N-ethylmaleimide-sensitive attachment receptor (SNARE) proteins necessary for exocytosis (13, 39), a role for CSP-α in the regulation of signaling through heterotrimeric GTP binding proteins (29, 31, 32), as well as a role for CSP-α in the regulation of transmembrane calcium flux (14, 23). Client proteins for CSP-α and Hsc70 chaperone activity in acinar cells have not been identified. Although Hsc70 is likely coupled to numerous folding processes in acinar cells, CSP-α almost certainly serves to anchor chaperone activity to ZGs and thereby targets Hsc70 and its stress-induced homolog Hsp70 to the secretory pathway.

In this study, we begin to address the hypothesis that the CSP-α/Hsc70 chaperone system is essential for maintaining the high fidelity of dynamic protein interactions necessary for the pancreatic acinar secretory response. As a first step toward testing this hypothesis, we evaluated the effects of introducing purified recombinant CSP-α truncation constructs into permeabilized acinar cells. Our findings demonstrate that CSP-α1-82 assemblies with Hsc70 or Hsp70 and significantly augments the Ca\(^{2+}\)-stimulated secretion of amylase by acinar cells. Furthermore, we demonstrate that, via residues 83-112, CSP-α associates with the vesicle associated membrane protein 8 (VAMP 8) present on ZG membranes. Our results demonstrate a role for CSP-α in the secretory pathway and implicate select acinar SNAREs as client proteins for the CSP-α/Hsc70 chaperone system.

**MATERIALS AND METHODS**

**Antibodies.** Polyclonal anti-VAMP 8 and anti-VAMP 2 monoclonal antibody c169.1, were purchased from Synaptic Systems. Chicken polyclonal VAMP 2 antibody was from Chemicon. Anti-Hsc70 and Hsp70 monoclonal antibodies were purchased from StressGen. Alexa-conjugated secondary antibodies were purchased from Molecular Probes. Peroxidase-conjugated sheep anti-mouse IgG and donkey anti-rabbit IgG were purchased from GE Healthcare. Anti-VAMP 8 polyclonal antibodies were generous gifts from T. Weimbs (28) and W. Hong (46). A polyclonal antibody to the cytosolic domain of recombinant human VAMP 8 was also produced in rabbits. All VAMP 8 antibodies gave essentially identical results for immunoblotting and immunofluorescence experiments. Two different anti-CSP polyclonal antibodies were utilized, a peptide antibody raised against the sequence CTQLTADSHPSYHTDGFN corresponding to amino acids 182-198 of rat CSP (5) and a second antibody raised against the sequence G1031CSP-\(\alpha\)/H9251l/137 NaCl, 4.7 KCl, 0.56 MgCl\(_2\), 1.28 CaCl\(_2\), 0.6 NaHPO\(_4\), 5.5 \(\beta\)-glucose, 2 t-glutamine, and an essential amino acid solution. The buffer was supplemented with 0.1 mg/ml soybean trypsin inhibitor and 1 mg/ml BSA, gassed with 100% O\(_2\), and adjusted to pH 7.48. Dispersed cultures of pancreatic acinar cells were isolated from adult male Sprague-Dawley rats by collagenase digestion as described previously (41). Acini were suspended in incubation buffer, and cells were maintained at 37°C for 1 h before performing assays. To induce Hsp70 expression, freshly harvested cells suspended in incubation buffer were incubated at 42°C for 30 min followed by a 2-h recovery at 37°C.

**Immunofluorescence microscopy.** Lobules were gently pelleted and fixed in 4% paraformaldehyde in PBS. Immunofluorescence microscopy was performed on 9-\(\mu\)-m-thick cryostat sections as detailed previously (42). VAMP 8 was detected with rabbit polyclonal antibodies raised against the cytosolic domain VAMP 8 from T. Weimbs (28), W. Hong (46), or by our laboratory (1:50). CSP-α was detected with rabbit polyclonal antibody raised against a CH\(_2\)-terminal peptide of the protein (1:50). VAMP 8 and CSP-α antibodies were detected using an Alexa 488-conjugated and Alexa 546-conjugated Zenon secondary detection kit, respectively. VAMP 2 was analyzed with a chicken polyclonal antibody (AB5625, 1:100) and an Alexa 488-conjugated anti-chicken IgG (1:250). When analyzed with VAMP 2, the CSP-α antibody (1:250) was detected with Alexa 488-conjugated anti-rabbit IgG (1:250). Fluorescence and differential interference contrast images were captured using a Nikon Eclipse TE2000 microscope, a PlanApo \(\times\) 100 oil objective with a numerical aperture of 1.4, and a Hamamatsu Orca camera. Stacks of Z-series images were deconvolved using Volocity software and processed using Volocity or Photoshop software. Calculations of fluorophore overlap were determined using Image J software.

**Acinar cell permeabilization.** Acini were suspended in a permeabilization buffer containing (in mM) 20 PIPES (pH 6.6), 139 K\(^+-\)glutamate, 4 EGTA, 1.78 MgCl\(_2\), 2 Mg-ATP, 0.1 mg/ml soybean trypsin inhibitor, 1 mg/ml bovine serum albumin, and 35 pM PFO. PFO is a cholesterol-dependent cytolysin that assembles to create large aqueous pores in cell membranes (24). PFO was allowed to bind to intact cells on ice for 15 min, and excess unbound PFO was then removed by being washed at 4°C in the same buffer without PFO. Acini were aliquoted into prechilled microcentrifuge tubes (200 \(\mu\)l/tube) containing the indicated amounts of recombinant proteins. The cell suspension was then diluted with an equal volume of the same buffer containing enough CaCl\(_2\) to create the desired final concentration of free Ca\(^{2+}\). The quantity of CaCl\(_2\) added to the buffer was calculated on the basis of dissociation constants using a computer program as previously described (41). Cell suspensions were immersed in a 37°C water bath and incubated with gentle mixing for the indicated times. To measure basal secretion, permeabilized cells were incubated with indicated amounts of recombinant proteins and/or 100 \(\mu\)M GTP-\(\gamma\)-S in the presence of \(\leq\)10 nM of free Ca\(^{2+}\). Following incubation, cells were cooled in an ice bath and then centrifuged at 12,000 \(\times\)g for 1 min. The content of amylase in the medium was determined using a Phadebas assay kit. Data were calculated as the percent of total cellular amylase present in an equal amount of cells measured at the start of the experiment.

**Preparation of ZGs.** Rat pancreases were minced in 5 vol of a buffer containing (in mM) 10 MOPS, pH 6.8, 250 sucrose, 0.1 MgCl\(_2\), 0.1 PMSF, and 1 benzamidine. Tissue was homogenized by 10 strokes of a motor-driven homogenizer (500 revolution/min) using a Teflon pestle with 0.5–1.0-mm clearance. A postnuclear supernatant was prepared by centrifugation at 1,000 \(\times\)g for 10 min and then further centrifuged at 3,200 \(\times\)g for 10 min to produce a white pellet enriched...
in ZGs overlaid by a brown pellet enriched in mitochondria. The remaining supernatant was centrifuged at 100,000 g for 1 h to separate microsomal and cytosolic fractions. ZGs were further purified by Percoll gradient centrifugation (42) and then lysed by sonication in buffer consisting of (in mM) 50 Tris (pH 7.4), 100 NaCl, 5 EDTA, 25 NaF, 10 Na pyrophosphate, and protease inhibitors. ZG membranes were then separated from content by 100,000 g centrifugation for 30 min. To remove peripherally associated proteins, ZG membranes were incubated in 0.1 M Na2CO3 (pH 11) for 30 min at 4°C and then recovered by centrifugation at 100,000 g for 1 h.

Pronase digestion of ZG proteins. To digest ZG surface proteins, 200-μl aliquots of intact Percoll-purified ZGs containing 1 mg protein were further diluted in 200 μl of buffer containing 50 mM MES, pH 5.5, 250 mM sucrose, 0.1 mM MgSO4 with or without 35 μg/ml of pronase. Following 10 min incubation on ice, 200 μl of a 100× protease inhibitor cocktail (Calbiochem Cat. No. 539131) containing AEBSF, aprotinin, E-64, EDTA, and leupeptin was added followed by dilution in SDS-PAGE buffer and boiling. Digestion of proteins on the interior of ZGs was conducted in the same manner except that ZGs were initially diluted in buffer containing both pronase and 1% Triton X-100 and then immediately sonicated before incubation on ice.

Glutathione S-transferase. Preparation of glutathione S-transferase (GST) fusion proteins of CSP and CSP truncation constructs were previously described (31). In all cases, GST was placed at the NH2 terminus of CSP. Likewise, the GST-VAMP 8 construct contains GST at the NH2 terminus. Fusion proteins were purified by glutathione affinity chromatography and either eluted in buffer containing 10 mM glutathione or released by thrombin cleavage as previously described (22).

GST pull-down assay and immunoprecipitations. Cell cytosol fractions or membrane fractions prepared from purified ZGs, solubilized in lysis buffer containing 1% TX-100, were incubated with 15 μg of GST alone or the recombinant GST-tagged proteins, together with 20 μl of glutathione-Sepharose beads at 4°C. After 1-h incubation at 4°C, the beads were washed extensively and fusion proteins eluted with SDS sample buffer. Binding proteins were analyzed by immunoblotting. For immunoprecipitations, detergent solubilized membrane fractions containing 1% Triton X-100 were incubated with indicated antibodies overnight at 4°C. Antibodies were then precipitated with Protein A-Sepharose beads for 1 h, washed extensively in lysis buffer, and analyzed by SDS-PAGE and immunoblotting.

RESULTS

CSP-α is present on the outer leaflet of ZGs in acinar cells. Three CSP genes have been identified; CSP-α, CSP-β and CSP-γ (18). CSP-β and CSP-γ are testis specific (18), whereas CSP-α, which is highly expressed in brain, was reported to undergo alternate splicing generating a truncated isoform in some cell lines (9). The expression of CSP-α in acini was demonstrated using two different polyclonal antibodies raised against either full-length rat CSP-α (1:1,000) (8) or a C-terminal sequence unique to full-length CSP-α (5). In accordance with previous work (5), both antibodies detected a diffuse 35-kDa CSP-α signal in acini attributable to multiple palmitoylation sites within the cysteine string region demonstrating that full-length CSP-α is the predominant form present in acinar cells (Fig. 1A).

Confirming previous studies (5, 42), tissue fractionation and immunoblotting demonstrated that CSP-α immunoreactivity was present in Percoll-purified ZG fractions of pancreas (Fig. 1B). Further fractionation of ZGs into ZG membranes and ZG content revealed a pronounced enrichment of CSP-α in the ZG membranes but no signal in ZG content. Moreover, Na2CO3 (pH 11) washing of ZG membranes to remove peripherally associated proteins further enriched the CSP-α signal, confirming that the molecule is embedded in the ZG membrane. Note that the faint CSP-α signal shown in the intact ZG fraction is due to the extremely short exposure time necessary to detect the protein in ZM and WZM fractions. Also note that CSP-α is not removed by Na2CO3 washing. C: purified ZGs were treated with or without pronase and Triton X-100 (T X-100) and either left intact or lysed by sonication to allow access of pronase to intragranular proteins. Proteins (35 μg/lane) from each fraction were separated by SDS-PAGE and analyzed by immunoblotting with anti-full-length CSP-α (1:1,000). Note that CSP-α is completely degraded by pronase treatment of intact granules, whereas GP-3, a protein on the inner surface of granules (45), remains intact, indicating that CSP-α is present on the cytoplasmic surface of ZG.
CSP-α interacts with acinar cell Hsc70 and Hsp70. To begin to evaluate a role for the CSP-α cochaperone system in acinar function, pull-down assays were conducted using GST-CSP-α1-198 representing full-length CSP-α or the truncated GST-CSP-α1-82 containing the J domain (see Fig. 6 for a diagram of the protein), and the assembly of CSP-α/Hsc70 and CSP-α/Hsp70 complexes in acinar lysates was evaluated (Fig. 2). Consistent with previous studies (44), acinar cells were found to constitutively express Hsc70 under normal conditions (Fig. 2, top), as well as to undergo an approximate 10-fold increase in the expression of stress-inducible Hsp70 in response to a 30-min heat shock at 42°C followed by a 2-h recovery (Fig. 2, middle and bottom). Both full-length CSP-α1-198 and CSP-α1-82 precipitated Hsc70 or Hsp70 from acinar lysates. Quantitatively, each construct precipitated less than 10% of total Hsc70 or Hsp70 in the lysate, which likely reflects the transient nature of this molecular interaction. These results are in agreement with previous reports using yeast two-hybrid and ATPase assay analysis (10, 39) demonstrating Hsp70 and Hsc70 association with CSP-α via the J domain and indicate that, in acinar cells, CSP-α/Hsc70 and CSP-α/Hsp70 may form functional chaperone complexes. CSP-α1-82 augments acinar secretion. Next the effect of introducing recombinant J domain (CSP-α1-82) into PFO-permeabilized acinar cells on Ca²⁺-stimulated amylase secretion was evaluated (Fig. 3A). Maximum amylase secretion from permeabilized cells was achieved at 3 μM free Ca²⁺ (data not shown). Thus isolated cells were permeabilized and incubated with the indicated concentrations of CSP-α1-82 and 3 μM Ca²⁺ for 30 min, and the release of amylase into the media was determined. CSP-α1-82 strongly enhanced Ca²⁺-stimulated secretion in a concentration-dependant manner with a maximal 180% of basal levels achieved at 200 μg/ml of protein. No further increase in secretion was detected at higher concentrations of CSP-α1-82 or with longer incubation times (data not shown). CSP-α1-82 had no effects on basal secretion, measured at ≤10 nM Ca²⁺. Unexpectedly, a CSP-α1-112 construct containing both the J domain and adjacent linker region had little or no effect on Ca²⁺-stimulated secretion when incubated at concentrations as high as 200 μg/ml (Fig. 3B). Similarly, introduction of full-length CSP-α1-198 also had no effects on Ca²⁺-stimulated secretion (data not shown), findings that potentially reflect the pronounced aggregating properties of the linker region of the molecule (see DISCUSSION). These results indicate that the NH₂-terminal region of CSP-α, which encodes the J-domain, is able to modulate Ca²⁺-dependent secretion of digestive enzymes and further suggest that the mechanism of this effect involves Hsc70 chaperone function. The J domain of CSP-α is comprised of four helices with a tripeptide of histidine, proline, and aspartic acid (HPD motif) located between helices II and III that is essential for activation of Hsc70 (52). If our hypothesis that CSP-α anchors chaperone work to zymogen granules and that this conformational work is critical to digestive enzyme secretion is correct, then mutation of the HPD motif to abolish its Hsc70 activating ability would be expected to eliminate the secretory effects of CSP-α1-82 when introduced into permeabilized acini. The histidine-proline-aspartate (HPD) sequence (amino acids 43-45) was mutated to glutamine-proline-alanine and introduced into the acinar cells (Fig. 3C). Consistent with our hypothesis, introduction of high concentrations (200 μg/ml) of CSP-α1-82HPD-QPA failed to augment Ca²⁺-stimulated secretion and modestly inhibited secretion by ~20%. These data support the concept that Hsc70 activation is essential in mediating the effects of CSP-α1-82 on Ca²⁺-stimulated secretion.

Similar to the present findings, overexpression of CSP-α in PC12 cells was previously shown to augment Ca²⁺-dependent secretion in permeabilized cells, indicating that CSP-α acts at a late step in the secretory pathway (11). Interestingly, this same study demonstrated that treatment of permeabilized cells overexpressing CSP-α with GTP-γS significantly enhanced basal secretion independent of elevated Ca²⁺. Incubation of permeabilized acini with GTP-γS enhanced basal secretion (in the presence of ≤10 nM free Ca²⁺) by ~155% of control values (Fig. 3D). Similar to results in PC12 cells (11), co-incubation of permeabilized acini with both CSP-α1-82 and GTP-γS further elevated basal secretion by 125% of that seen for GTP-γS alone. These results suggest that CSP-α may play a role in regulating the activity of G proteins that are important for the secretory response.

CSP-α colocalizes with VAMP proteins in the secretory pathway of acinar cells. To further evaluate the subcellular localization of CSP-α in acini, immunofluorescence analysis was conducted on cryostat sections of pancreatic lobules (Fig. 4), confirming that the protein is abundant in apical ZG-rich regions of the cytoplasm. We recently reported that acinar cells of the pancreas contain at least 2 populations of ZGs on the basis of their expression of VAMP 2 or VAMP 8 (48). Thus dual immunofluorescence and quantitative analysis of CSP-α and VAMP proteins was conducted to determine the VAMP-specific localization of CSP-α. Results indicated a 52.1 ± 5.0% (n = 10) colocalization of CSP-α with VAMP 8 in apical

Fig. 2. The CSP-α J domain interacts with acinar cell Hsc70 and Hsp70. Isolated acini were incubated under control conditions at 37°C (top) or heat shocked at 42°C for 30 min (middle and bottom) and then returned to 37°C for an additional 120 min to induce Hsp70 expression. Lysates from control (Con) or heat-shocked (HS) acini were incubated with purified glutathione S-transferase (GST), GST-CSP-α1-82 containing the J domain, or full-length GST-CSP-α1-198 as indicated. Bound proteins were analyzed by immunoblotting (IB) using Hsc70 (1:600) or Hsp70 (1:300) specific antibodies. Note the large induction of Hsp70 by heat shock. Also note that the CSP-α1-82 and CSP-α1-198 coprecipitate both Hsc70 and Hsp70.
regions of acini. In contrast, CSP-α showed a more modest 36.2 ± 2.8% (n = 10) colocalization with VAMP 2. Similarly, immunofluorescence analysis of cryostat sections prepared from Percoll-purified ZG granules supported these findings demonstrating a more extensive colocalization of CSP-α with VAMP 8 compared with VAMP 2 (Fig. 5). These data confirm the tissue fractionation experiments localizing CSP-α to ZGs (5, 42) and further show that CSP-α significantly colocalizes with VAMP 8-containing ZGs.

**CSP-α-SNARE interactions in acinar cells.** CSP-α has been shown to interact with a number of SNARE proteins including syntaxins 1A (34) and 4 (12), synaptotagmin 1 (16), as well as some isoforms of VAMPs (4). We were unable to detect interactions between CSP-α and the plasma membrane SNARE proteins SNAP 23, syntaxin 2, or syntaxin 4 when analyzed by coimmunoprecipitation from microsomal fractions of acini (data not shown). However, it has been our experience that coimmunoprecipitation of SNARE proteins in acini yield extremely small quantities of mature SNAREpin complexes, making these difficult to detect (48). As an alternative, potential CSP-α interactions with various SNARE proteins expressed on ZGs were examined in Triton X-100-solubilized ZG membrane fractions by coimmunoprecipitation with anti-CSP-α antibodies (Fig. 6A). Consistent with the colocalization of CSP-α and VAMP 8 in acini and ZG fractions, these proteins coimmunoprecipitated from the ZG membrane fraction. Conversely, neither VAMP 2 nor syntaxin 3, which are also expressed on ZGs (20), was detected with CSP-α precipitated protein (syntaxin 3 is not shown). The interaction between CSP-α and VAMP 8 was further confirmed by pull-down assays where GST-VAMP 8 precipitated CSP-α (Fig. 6B) and GST-CSP-α precipitated VAMP 8 (Fig. 6C) from ZG membranes.

The CSP-α linker region is required for VAMP 8 interactions. The molecular interaction between CSP-α and VAMP 8 was characterized using GST-CSP-α truncation constructs (Fig. 7). Neither CSP-α1-82 nor CSP-α137-198 was found to interact with VAMP 8 in Triton X-100-soluble ZG membrane fractions. In contrast, CSP-α1-112 and CSP-α83-198 interacted with VAMP 8. Compared with full-length CSP-α1-198, which pulled down 12.5 ± 7.8% of the total VAMP 8 in the fraction, the CSP-α1-82 and CSP-α137-198 pulled down 5.1 ± 1.6% and 6.0 ± 0.4% of total VAMP 8, respectively; however, these differences were not statistically significant (n = 3). None of the CSP-α constructs tested interacted with VAMP 2. Because CSP-α1-112 bound to VAMP 8, whereas CSP-α1-82 did not, these data indicate that the linker region (amino acids 83-112) is capable of VAMP 8 binding. Results that the COOH-terminal construct CSP-α137-198 showed no binding activity, whereas CSP-α83-198 bound to VAMP 8, confirmed the significance of the linker region in binding; however, the overall importance of the cysteine string region (amino acids 113-137) in VAMP 8 binding was not determined.
DISCUSSION

The present study establishes that CSP-α1-82 encoding the signature J domain augments Ca^{2+}-induced digestive enzyme secretion when introduced into permeabilized acini. CSP-α1-82 is soluble, and we anticipate that cytosolic CSP-α1-82 when introduced into permeabilized cells is able to mimic acinar folding events carried out by native ZG-anchored CSP-α. A major question that remains is whether the action of CSP-α1-82 is interchangeable with respect to other J domain proteins that may be expressed in acinar cells. Sequence analysis has revealed over 40 J domain proteins; however, to date, functional roles for only a few of these molecules have been demonstrated (52). The specificity of the J domain-Hsc/Hsp70 protein folding apparatus appears to be based largely on the select subcellular distribution of J proteins that act to target Hsc/Hsp70 chaperone activity in the cell. Clearly, our demonstration that CSP-α localizes to the outer leaflet of ZGs and undergoes a direct interaction with VAMP 8 strongly supports that this chaperone complex acts to promote conformational changes in the exocytic protein machinery of the acinar cell necessary for maintaining a robust secretory response.

A functional role for CSP-α in regulated secretion has been demonstrated in neuronal, neuroendocrine, and endocrine cell types revealing several potential mechanisms for the protein including modulation of voltage-regulated Ca^{2+} channels, neurotransmitter synthesis, vesicle filling, regulation of heterotrimeric G proteins, and phosphorylation-dependent conformational changes in exocytic SNARE proteins (reviewed in 17, 18). Drosophila CSP-α-null mutants exhibit temperature-sensitive paralysis, and most flies die as larvae or within days of adulthood (53). Likewise, deletion of CSP-α in mice causes blindness and progressive neurodegeneration with no survival beyond 4 mo (18, 37). Compared with neural and endocrine secretory models, the acinar cell has a number of unique features. Secretion does not involve voltage-dependent Ca^{2+} influx but is mediated via G protein-coupled receptors and the IP_{3}-dependent release of Ca^{2+} from internal stores (49). Moreover, secretion...
involves the sequential compound exocytosis of ZGs following an initial round of ZG fusion to the apical plasma membrane (33), and this process is regulated by unique SNARE protein isoforms (46, 48 and reviewed in 49). Thus it is probable that CSP-α function in acini may vary with respect to its effects on Ca\(^{2+}\) signaling and SNARE isoform interactions seen in neural and endocrine systems.

Results that CSP-α\(_{1-82}\) modulates Ca\(^{2+}\)-stimulated exocytosis in permeabilized acini independent of hormone/neurotransmitter activation are clearly in line with the demonstrated role for CSP-α in other secretory models, indicating that the protein acts at a late stage in the exocytic process (reviewed in 17). Similar to our findings, Chamberlin et al. (11) stably overexpressed CSP-α in PC12 cells, demonstrating that it augmented Ca\(^{2+}\)-stimulated exocytosis from permeabilized cells. When overexpressed, CSP-α was shown to localize correctly within the cells and had no effects on cell morphology, granule distribution, and number or intracellular Ca\(^{2+}\)-signaling, indicating that the protein was acting at a late stage in the secretory pathway. Conversely, transient overexpression of CSP-α in β-cells (8, 50), HIT-T15 cells (51), or chromaffin cells (21) inhibited secretion. Interestingly, Boal et al. (4) confirmed that CSP-α overexpression in HIT-T15 cells inhibited insulin secretion from intact cells, whereas CSP-α\(_{1-82}\) containing only the J domain had no inhibitory effects. Rather, the inhibitory effects of CSP-α with transient overexpression were mapped to the central linker and COOH-terminal regions of the molecule. These findings confirmed earlier results from Zhang et al. (51) demonstrating that mutation of glutamate 93 to valine within the linker region significantly reduced the inhibitory effects of the molecule on secretion. Finally, and in contrast to the present results, overexpression of the CSP J domain in Xenopus oocytes inhibited phorbol-ester-induced cortical granule exocytosis, and this effect was abolished by mutation of the J domain to interfere with Hsc70 binding (39). The wide variability of these secretory effects caused by overexpressing CSP-α in cells is no doubt related to the experimental conditions utilized in each study including cell type, transient or stable transfections, permeabilized vs. intact cells, and the nature of the secretory stimulus.

Fig. 5. CSP-α colocalizes with VAMP-8 on purified ZGs. ZGs were isolated by Percoll-density centrifugation and then fixed in 4%-formaldehyde. Cryostat sections were analyzed as in Fig 3. Note that CSP-α is more highly colocalized with VAMP 8 compared with VAMP 2.
Nie et al. (34) demonstrated that CSP overexpression in secretory proteins was rescued by coexpressing syntaxin 1A, which directly interacts with CSP. These results suggest that, when introduced into permeabilized cells during Ca\(^{2+}\) stimulation, CSP may interact with or otherwise sequester important components of the SNARE apparatus, thereby inhibiting exocytosis. These interactions may not be as extensive when the molecule is acutely introduced into permeabilized cells during Ca\(^{2+}\) stimulation. Under these conditions the linker region may be unable to acutely interact with regulatory molecules (including SNARE proteins) when ZGs are docked and/or poised for secretion. A second possibility is that recombinant CSP-\(\alpha\) is known to form high molecular mass SDS-resistant oligomers and aggregates following purification from bacterial lysates (1, 40). Moreover, this self-association of CSP-\(\alpha\) was mapped to the linker and cysteine string domains (amino acids 83-136) of the protein (40). Thus it is conceivable that the high molecular mass CSP-\(\alpha\) oligomers that form during purification are unable to gain sufficient access into PFO-permeabilized cells compared with the highly soluble CSP-\(\alpha_{1-82}\) J domain. Whether or not this aggregation property of CSP-\(\alpha\) plays a role in inhibiting secretion when the protein is overexpressed at high levels in cells is not certain.

Coimmunoprecipitation and GST pull-down assays have established that CSP-\(\alpha\) directly interacts with syntaxin 1A (34),...
syntaxin 4 (12), synaptotagmin 1 (16), VAMP 2, and VAMP 7, but not VAMP 3 (4). Interestingly, the interaction of GST-
CSP-α with VAMP 2 was dependent on the presence of Ca²⁺
and appeared to require the C-terminal amino acids of the
protein because truncation of these residues (representing the
alternatively spliced isoform CSP2) showed no binding activity
(4). In the present study, no interaction between CSP-α and
VAMP 2 was detected in Triton X-100 solubilized ZG mem-
brane fractions. Furthermore, inclusion of Ca²⁺ in the buffer
did not promote a VAMP 2 interaction with CSP-α or enhance
its association with VAMP 8 (data not shown). A lack of
VAMP 2 binding may potentially reflect the low levels of
CSP-α and VAMP 2 colocalization in acini; rather, our results
indicate that CSP-α interacts with VAMP 8 via the linker
region, whereas the C-terminal construct (CSP137-198) showed
no binding activity (see Fig. 6). The differences for these
results are uncertain; however, the lack of CSP-α association
with VAMP 2 is clearly supported by the more modest colo-
calization of these molecules compared with VAMP 8.

Prior induction of Hsp70 in isolated cells and rodents has
been shown to provide a substantial protective effect against
the onset of acute pancreatitis (2, 44), and Hsp70-deficient
cells display increased sensitivity to the disease (25). The
cascade of events that underlie acute pancreatitis and the
protective effects of Hsp70 remain to be identified but appear
to involve aberrant alterations in the acinar secretory pathway
(27). Acute pancreatitis is experimentally induced by admin-
istering supraphysiological levels of secretagogues to rodents
or isolated acinar cells. The earliest events seen within minutes
of stimulation involve the inhibition of apical secretion, exocyto-
sis of ZGs at the basolateral membrane, premature activation of
trypsinogen within cells, and the formation of large cytoplas-
mic vacuoles (reviewed in 27). Further evidence implicating
the acinar secretory pathway in the pathogenesis of acute
pancreatitis comes from the finding that genetic deletion of
the ZG protein VAMP 8 significantly reduces the severity of
experimental pancreatitis in rodents (15, 46). Thus it is tempt-
ing to speculate that, just as CSP-α has been implicated as an
important factor in attenuating neurodegenerative disease (18),
it may also play a significant role in maintaining the overall
integrity of the secretory pathway and, in the presence of high
levels of Hsp70, protect against the onset of acute pancreatitis.
Clearly further investigation of a potential role for CSP-α in
mediating the protective effects of Hsp70 against acute pan-
creatitis may provide valuable mechanistic insight into the
pathophysiology of this disease as well as potential targets for
therapeutic intervention.

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CSP-α REGULATES ACINAR SECRETION


