RESEARCH COMMUNICATION

Phosphorylation-dependent interaction of the synaptic vesicle proteins cysteine string protein and synaptotagmin I

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The secretory vesicle cysteine string proteins (CSPs) are members of the DnaJ family of chaperones, and function at late stages of Ca^{2+}-regulated exocytosis by an unknown mechanism. To determine novel binding partners of CSPs, we employed a pull-down strategy from purified rat brain membrane or cytosolic proteins using recombinant hexahistidine-tagged (His_6-)CSP. Western blotting of the CSP-binding proteins identified synaptotagmin I to be a putative binding partner. Furthermore, pull-down assays using cAMP-dependent protein kinase (PKA)-phosphorylated CSP recovered significantly less synaptotagmin. Complexes containing CSP and synaptotagmin were immunoprecipitated from rat brain membranes, further suggesting that these proteins interact *in vivo*. Binding assays *in vitro* using recombinant proteins confirmed a direct interaction between the two proteins and demonstrated that the PKA-phosphorylated form of CSP binds synaptotagmin with approximately an order of magnitude lower affinity than the non-phosphorylated form. Genetic studies have implicated each of these proteins in the Ca^{2+}-dependency of exocytosis and, since CSP does not bind Ca^{2+}, this novel interaction might explain the Ca^{2+}-dependent actions of CSP.

Key words: exocytosis, Hsc70, protein kinase A, protein phosphorylation.

INTRODUCTION

Cysteine string proteins (CSPs) are ubiquitously expressed secretory vesicle membrane proteins first discovered in *Drosophila* [1]. The characterized domains of CSP are: (i) its defining cysteine string motif (a stretch of 20 amino acids containing 14 cysteine residues), which is palmitoylated and targets the protein to secretory vesicles [2]; (ii) an N-terminal J-domain, making it a member of the DnaJ family of co-chaperones that binds Hsc70 (heat-shock cognate protein of 70 kDa) [3,4]; and (iii) a linker region between the J-domain and the cysteine string found to be important for exocytosis [5]. *In vitro*, CSP can recruit Hsc70 and stimulate its ATPase activity to refold denatured polypeptides [6], an activity that is enhanced further by the binding of a novel, small, glutamine-rich co-chaperone containing three tandem tetratricopeptide repeats, SGT [7]. This chaperone activity has not been observed as an *in vivo* function of CSP; however, on the basis of its biochemical properties, CSP has been dubbed a 'chaperone of the synapse' [7–9].

In vivo, it is clear that CSP is required for viability and is an important component of the exocytotic apparatus, since null mutations in mice and *Drosophila* are generally lethal [7,10] and, in *Drosophila*, the few surviving CSP null mutants have a reduced Ca^{2+}-sensitivity of neurotransmitter release, downstream of Ca^{2+} entry [10, 11]. Further support for a role of CSP in the late stages of exocytosis has originated from observations that amperometric spikes measured in permeabilized adrenal chromaffin cells over-expressing CSP have altered kinetics, indicative of slowed fusion pore opening [12]. We have recently shown that this effect is dependent upon phosphorylation of CSP on Ser^{10} [13]. In addition, CSP has a genetic interaction with the soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) protein syntaxin in *Drosophila*, which has been confirmed biochemically for the mammalian proteins [10,13,14]. Interestingly, Hsc70-4 (the closest homologue to mammalian Hsc70 in *Drosophila*)-defective mutants have a Ca^{2+}-dependent phenotype that is similar to that of CSP mutants [15]. The Ca^{2+}-sensitive phenotype of CSP and Hsc70-4 mutant flies lies downstream of Ca^{2+} entry, and thus cannot be explained by the additionally characterized effects of CSP upon voltage-dependent Ca^{2+} channels [16–19]. Furthermore, no Ca^{2+} binding activity has been attributed previously to CSP, Hsc70 or SGT. Taken together, this evidence suggests that CSP may modulate the formation of exocytotic protein complexes involved in late Ca^{2+}-dependent stages of vesicle fusion as part of a multimeric chaperone complex. In view of this, we employed a pull-down assay to detect new targets for CSP. Synaptotagmin I, the putative Ca^{2+} sensor for exocytosis [20, 21], was identified as a novel CSP-binding protein. Unlike previously characterized protein interactions of CSP, including that with syntaxin [10] and G-protein $\beta\gamma$ subunits [16], the interaction with synaptotagmin might provide the missing link between CSP function and the coupling of Ca^{2+} to exocytosis.

EXPERIMENTAL

Materials

CSP antiserum was as previously described [22]. Anti-(synaptotagmin I) monoclonal antibody was from Synaptic Systems (Göttingen, Germany). Recombinant hexahistidine-tagged (His_6-) CSP was expressed and purified as described previously [4]. The pGEX-synaptotagmin I-(120–380) construct [containing the C2A and C2B domains of synaptotagmin (amino acids 120–

Abbreviations used: CSP, cysteine string protein; Hsc70, heat-shock cognate protein of 70 kDa; PKA, cAMP-dependent protein kinase; GST, glutathione S-transferase; S10A, CSP mutant bearing the site-directed replacement of Ser^{10} with alanine; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor.

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380)] was generously provided by Dr V. Haucke (University of Göttingen, Göttingen, Germany) and glutathione-S-transferase (GST)–synaptotagmin I-(120–380) was expressed and purified as described previously [23]. Ni^{2+}-agarose was obtained from Qiagen (Crawley, West Sussex, U.K.). Protein G and glutathione–Sepharose FF beads were from Amersham Biosciences (Little Chalfont, Bucks., U.K.). cAMP-dependent protein kinase (PKA) catalytic subunit, anti-Hsc70 monoclonal antibody and all other reagents were from Sigma (Poole, Dorset, U.K.).

Phosphorylation of His_6-CSP

His_6-CSP was incubated in the presence or absence (mock) of PKA, according to a protocol described previously [13].

Pull-down assays

The pull-down of CSP-binding proteins from rat brain membrane and cytosol preparations was modified from a method published previously [16]. Rat brain membrane and cytosol proteins were prepared, also as described previously [24]. Briefly, 2.3 μg of mock- or PKA-phosphorylated His_6-CSP was incubated with 200 μg of rat brain membrane or cytosol protein and 20 μl of Ni^{2+}–agarose in binding buffer [20 mM Mops (pH 7.0)/4.5 mM $MgCl_2$/150 mM NaCl/0.5 % (v/v) Triton X-100/50 mM imidazole] in a total volume of 100 μl for 2 h, end-over-end at 4 °C. The Ni^{2+}–agarose was then washed three times in binding buffer, and CSP–protein complexes were eluted with SDS sample buffer [4 % (w/v) SDS/20 % (v/v) glycerol/10 % (v/v) 2-mercaptoethanol/ 0.04 % Bromophenol Blue and 125 mM Tris/HCl (pH 6.8)]. One-third of the eluted proteins and 5 % of the pull-down supernatant were separated on SDS/polyacrylamide gels, and transferred on to a nitrocellulose membrane. CSP, Hsc70 and synaptotagmin were detected by immunoblotting with specific antibodies.

In vitro binding assays

The *in vitro* binding of His_6-CSP to GST–synaptotagmin I-(120–380) was performed essentially as described previously for His_6-CSP and GST–syntaxin [14]. For determination of the affinity of the interaction, 0.5 μM GST–synaptotagmin I-(120–380) and a range (between 0 and 1 μM) of concentrations of mock- or PKA-phosphorylated His_6-CSP were used. Because the chemiluminescence detection system has a narrow linear range and a wide range of protein concentrations were used in the binding assays, a ^{125}I-labelled anti-rabbit IgG secondary antibody was used for CSP immunoblotting. ^{125}I-labelling of immunoblots was determined by exposure to a Phosphorscreen, and subsequent analysis using a Molecular Dynamics PhosphorImager with ImageQuant software (Sunnyvale, CA, U.S.A.). A logistic curve was fitted to the His_6-CSP data using SigmaPlot 2000 software, allowing calculation of an EC_{50} for CSP binding to synaptotagmin.

Immunoprecipitation

Immunoprecipitation of CSP from rat brain membranes was performed as described previously [25] using 2 μl of CSP antiserum and 200 μg of rat brain membrane protein. One third of the immunoprecipitates and 5 % of the supernatants were analysed for synaptotagmin immunoreactivity by Western blotting.

RESULTS

To discover novel binding partners of CSP, we employed a pull-down assay from rat brain proteins using recombinant His_6-CSP. To ensure that the assay was specific for proteins forming complexes with CSP, we probed the recovered proteins with an antibody raised against Hsc70, a well-established binding partner of CSP [3,4] and a protein well recognized for binding non-specifically to immobilized resins. Hsc70 was specifically contained in the His_6-CSP pull-down from rat brain cytosol, and no Hsc70 was detected bound to Ni^{2+}–agarose in the absence of CSP (Figure 1A). Other known binding partners of CSP, including G-protein G_β subunits [16], were also specifically bound to His_6-CSP in this assay (results not shown). Subsequent screening of the His_6-CSP-binding proteins with antibodies raised against various exocytotic proteins revealed highly detectable amounts of synaptotagmin I specifically captured by His_6-CSP from the rat brain membranes (Figure 1B, lane 'CSP'). We have reported previously that CSP is phosphorylated on Ser^{10} by PKA both *in vitro* and *in vivo*, and that phosphorylated CSP binds syntaxin with 10-fold-lower affinity than dephosphorylated CSP [13]. To determine the effect of CSP phosphorylation upon its interaction with synaptotagmin, the pull-down assay was performed using PKA-phosphorylated His_6-CSP. Figure 1(B), lane 'P-CSP' demonstrates the markedly decreased recovery of synaptotagmin with phosphorylated CSP compared with that of non-phosphorylated CSP. In contrast, detection of Hsc70 by immunoblotting revealed equivalent binding of Hsc70 to non-phosphorylated CSP and phosphorylated CSP, demonstrating the specificity of the phosphorylation-dependent interaction of CSP and synaptotagmin (Figure 1B). To address the quesion of whether endogenous CSP–synaptotagmin complexes exist

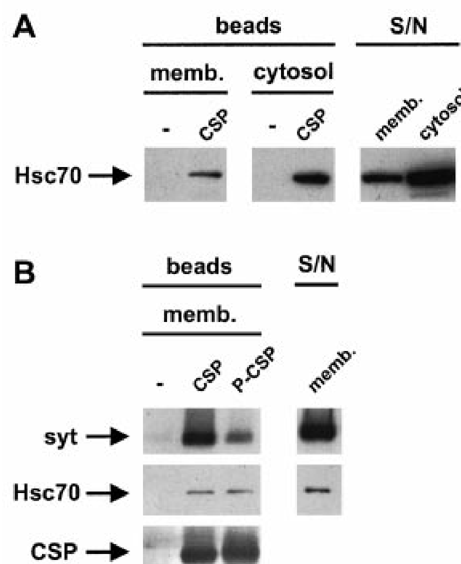

Figure 1 CSP pull-down recovers synaptotagmin

(**A**) Recombinant His_6-CSP or (**B**) His_6-CSP or PKA-phosphorylated His_6-CSP were immobilized on Ni^{2+}–agarose and incubated with rat brain membrane or cytosol proteins. Proteins bound to the beads and 5% of the supernatant (S/N) were immunoblotted with antibodies raised against (**A**) Hsc70 or (**B**) Hsc70 and synaptotagmin I (syt). A portion of the recovered proteins were separated on gels, and subjected to silver staining to ensure equal loading of His_6-CSP and PKA-phosphorylated His_6-CSP.

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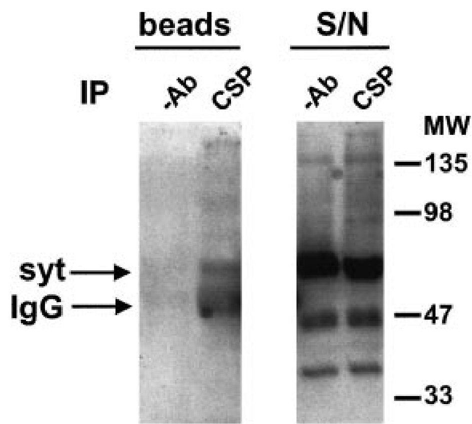

Figure 2 Co-immunoprecipitation of CSP and synaptotagmin

Rat brain membranes were incubated in the presence (CSP) or absence (—Ab) of anti-CSP rabbit polyclonal antiserum. The co-immunoprecipitated proteins and 5% of the supernatant (S/N) were immunoblotted with mouse monoclonal anti-synaptotagmin I antibodies by Western blotting (syt). Note the non-specific binding of the anti-mouse secondary antibody to the anti-CSP IgG heavy chain (IgG). Data shown are representative of three separate experiments. Molecular-mass ('MW') markers (in kDa) are indicated on the right.

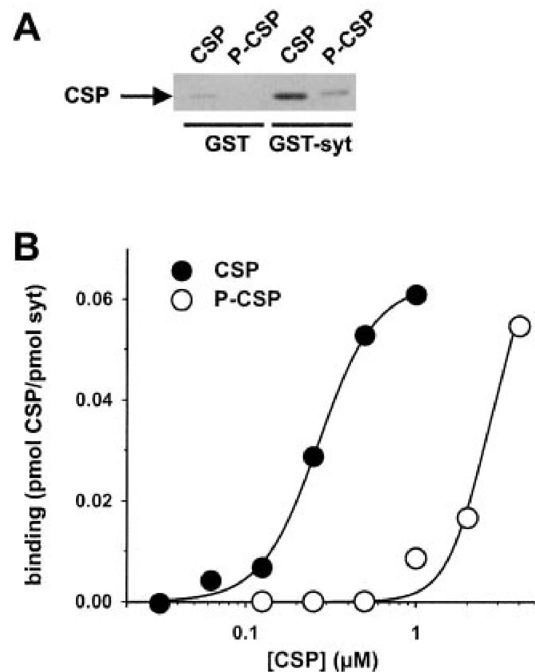

Figure 3 Direct binding of CSP to synaptotagmin *in vitro*

(**A**) GST–synaptotagmin I-(120–380) or GST (0.5 μM) were immobilized on glutathione–Sepharose beads and incubated with His_6-CSP or PKA-phosphorylated His_6-CSP (0.5 μM). After thorough washing, proteins bound to the beads were eluted with SDS sample buffer, and the recovered CSP was detected by immunoblotting. (**B**) The specified concentrations of His_6-CSP or PKA-phosphorylated His_6-CSP were used in the GST–synaptotagmin binding assay. The Western blotting protocol incorporated a ^{125}I-labelled anti-rabbit secondary antibody incubation and subsequent exposure of the blot to a Phosphorscreen to ensure linear detection of CSP across a wide range of concentrations. Densitometry data for the amount of CSP bound was normalized to the amounts of synaptotagmin and CSP added to each tube, and the small amounts of CSP that bound GST alone (above 0.5 μM CSP) were subtracted. The molar amounts of His_6-CSP bound for each concentration were then calculated by comparison with known amounts of His_6-CSP run on the same gel. The data were fitted to a three-parameter logistic equation, yielding an EC_{50} of approx. 0.25 μM. Even at 4 μM, binding of PKA-phosphorylated His_6-CSP to synaptotagmin was not saturated; however, the EC_{50} was estimated to be approx. 2.5 μM. The data shown are representative of three separate experiments.

in vivo, we performed immunoprecipitation from rat brain membrane proteins in the presence or absence of CSP antiserum. Immunoblotting of the immunoprecipitates with anti-synaptotagmin antibodies gave a readily detectable amount of synaptotagmin co-precipitated with CSP (Figure 2).

The pull-down and immunoprecipitation data suggest CSP and synaptotagmin can exist together in complexes, but do not address whether the proteins interact directly, or indirectly via one or more proteins in a multipartite complex. The latter is a distinct possibility, considering that CSP binds syntaxin, an established binding partner of synaptotagmin [26]. To test whether CSP can bind synaptotagmin directly, an *in vitro* GST pull-down assay using recombinant GST–synaptotagmin-I domain C2AB (amino acids 120–380) and various concentrations of phosphorylated or non-phosphorylated His_6-CSP was employed. The synaptotagmin construct containing the C2A and C2B domains was used because they are involved in most of the protein's interactions. CSP binding was detected by immunoblotting, and ^{125}I-labelled secondary antibodies were used to ensure linear quantification of binding. Direct, high-affinity binding of CSP to synaptotagmin was observed, with an EC_{50} of approx. 0.25 μM for the dephosphorylated form (Figure 3). In line with the His_6-CSP pull-down experiment, the phosphorylated form of CSP bound minimally to synaptotagmin, with an estimated EC_{50} of 2.5 μM, shifting the binding curve approximately one order of magnitude to the right.

Synaptotagmin is a Ca^{2+}-binding protein, and many of its protein–protein interactions, such as oligomerization, binding phospholipids, syntaxin, 25 kDa synaptosome-associated protein ('SNAP-25'), assembled SNARE complex and synaptic vesicle protein 2 ('SV2'), are Ca^{2+}-dependent [21,23,26–28]. We tested the Ca^{2+}-dependency of the CSP–synaptotagmin interaction using fixed concentrations of His_6-CSP and GST–synaptotagmin in the presence of 0–1 mM free Ca^{2+}, tightly buffered by EGTA and nitrilotriacetate. However, there were no significant differences in CSP–synaptotagmin binding over the entire range of Ca^{2+} concentrations studied (results not shown).

DISCUSSION

We have described a novel interaction between two synaptic vesicle proteins, CSP and synaptotagmin, both of which have fundamental Ca^{2+}-dependent roles in neurotransmitter exocytosis, as demonstrated by genetic experiments [10,11,20,29]. It is perhaps surprising that this interaction has not been observed previously, considering the intensive research synaptotagmin has received in recent years. We have shown that CSP and synaptotagmin are present in complexes in rat brain and, furthermore, we have demonstrated that they can bind each other directly *in vitro* in a phosphorylation-dependent manner. Indeed, the phospho-dependency of this interaction might account for its previous lack of detection. Since phosphorylated CSP binds synaptotagmin with a very low affinity, the interaction would be difficult to detect in synaptotagmin pull-down or immunoprecipitation experiments if the majority of endogenous CSP was phosphorylated. In support of this, we found previously a high phosphorylation of CSP under basal conditions in rat brain synaptosomes [13]. By biasing the phosphorylation state of CSP to 100%-dephosphorylated using His_6-CSP in the pull-down

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assay, we recovered readily detectable amounts of endogenous synaptotagmin. In addition, we immunoprecipitated endogenous synaptotagmin with our CSP antibody; however, immunoprecipitation with a commonly used anti-synaptotagmin monoclonal antibody (clone 41.1, raised against the linker and C2A domain) immunoprecipitated almost all of the synaptotagmin from the input sample, and no CSP was detected (results not shown). Since we have shown that CSP can bind the C2AB domain of synaptotagmin *in vitro*, it is possible that the synaptotagmin antibody binds epitopes that are masked in CSP–synaptotagmin complexes. With regard to the binding site for synaptotagmin on CSP, we can conclude from the phosphodependency of the interaction that the N-terminal amino acids of CSP containing the Ser^{10} phosphorylation site participate directly in or, at the very least, modulate the interaction. In support of this, we have found that mutation of Ser^{10} to Ala^{10} (S10A) results in disruption of CSP–synaptotagmin binding (results not shown). Since the S10A mutation still permits CSP binding to syntaxin [13], these data highlight the importance of the tertiary structure of the CSP N-terminus for the CSP–synaptotagmin interaction.

If, as predicted by the *in vitro* biochemical properties of CSP, its physiological function is to act as a co-chaperone in secretory cells, it is likely that the interactions of CSP with Hsc70 and SGT are important for its function as part of a chaperone complex, but will not specifically define its function in exocytosis [7]. Therefore other exocytotic proteins that CSP interacts with will be the targets of CSP action in exocytosis. Thus far, in addition to Hsc70 and SGT, CSP has been shown to directly bind syntaxin and G-protein $\beta\gamma$-subunits *in vivo* [10,13,14,16,30]. An attractive hypothesis is that the CSP–Hsc70–SGT complex acts as a chaperone for the SNARE complex via an interaction with syntaxin, in line with a role for CSP in the late stages of vesicle fusion [10,12,13]. However, *Drosophila* CSP null mutants have decreased evoked neurotransmission that is caused by the reduced efficiency of Ca^{2+} to trigger exocytosis downstream of Ca^{2+} influx [11]. This Ca^{2+}-dependent phenotype is strikingly similar to the phenotypes of synaptotagmin mutants in *Drosophila* [31], *Caenorhabditis elegans* [32] and a recent knock-in study in mice, where a mutation causing reduced Ca^{2+} affinity of synaptotagmin proportionally reduced the Ca^{2+}-sensitivity of neurotransmission [20]. Moreover, this reduced Ca^{2+}-sensitivity of exocytosis phenotype is shared by mutants of the *Drosophila* homologue of Hsc70 (Hsc70-4) [15]. Thus, with an addition to the model whereby a CSP-containing chaperone complex also orchestrates interactions between synaptotagmin and its exocytotic binding partners, the Ca^{2+}-binding function of synaptotagmin could account for the effect of CSP mutants upon the Ca^{2+}-sensitivity of neurotransmission.

In addition to the similarities in the Ca^{2+}-dependency of CSP and synaptotagmin mutants, CSP overexpression in adrenal chromaffin cells and synaptotagmin overexpression in PC12 cells both result in changes to initial amperometric spike kinetics, interpreted as prolonged fusion pore opening [12,13,33]. Of the exocytotic proteins tested in such systems, only CSP or synaptotagmin produce these effects and, interestingly, cells treated with agents that increase PKA activity have similar amperometric spike kinetics [34]. We have previously shown that the effects of CSP upon prolonged fusion pore opening require Ser^{10} [13], and phosphorylation or mutation of this residue reduces binding to synaptotagmin. Thus the phosphorylation state of CSP, determined by the activity of PKA, could modulate the availability of synaptotagmin for partaking in interactions that regulate fusion pore opening.

In addition to its established role in exocytosis, synaptotagmin has been implicated in endocytosis as a result of genetic experiments in *Drosophila* and *C. elegans*, and on the basis of its binding to the clathrin adaptor complex AP-2 and the stoned proteins [35–37]. However, we predict that the function of a CSP–synaptotagmin interaction would be relevant only to exocytosis, since CSP *Drosophila* mutants do not have deficiencies in endocytosis [38]. The same is true for CSP–Hsc70 interactions, since Hsc70 is involved in exo- and endo-cytosis, and auxilin is the co-chaperone for recruiting Hsc70 in endocytic clathrin uncoating [15,39]. Thus, for synaptotagmin and Hsc70, binding to CSP might confer specificity for functions in exocytosis, as opposed to endocytosis.

One of the daunting aspects of determining the molecular mechanism of regulated exocytosis is that many of the key proteins involved have numerous putative binding partners, and the elucidation of which interactions are functionally important at which stage is required. The finding that two major exocytotic proteins, syntaxin and now synaptotagmin, both bind CSP, part of a putative synaptic chaperone complex, might explain how their myriad interactions are tightly regulated. In addition, our discovery of an interaction between CSP and synaptotagmin offers the first molecular explanation for how CSP might be involved in the coupling of Ca^{2+} to secretion.

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