RESEARCH COMMUNICATION

The cysteine-string domain of the secretory vesicle cysteine-string protein is required for membrane targeting

Luke H. CHAMBERLAIN and Robert D. BURGOYNE¹

The Physiological Laboratory, University of Liverpool, Crown Street, Liverpool L69 3BX, U.K.

The post-translational addition of palmitic acid residues to cysteine-string protein (Csp) was originally thought to form the basis for membrane association of this secretory-vesicle protein. However, subsequent work showed that chemical depalmitoylation of Csp does not result in its release from membranes. We have confirmed these findings and employed [³H]palmitate labelling of PC12 cells to demonstrate that Csp1 remains associated with membranes following the complete removal of palmitic acid residues. Although palmitoylation is not essential for the stable

membrane association of Csp, its role in membrane targeting has not been assessed. To examine this, we constructed a Csp mutant protein with seven cysteines replaced by serines in the cysteine-string domain. In contrast to wild-type Csps, this mutant protein was not targeted to membranes when expressed in PC12 or HeLa cells. We conclude that although a palmitoylated cysteine-string domain is not required for stable membrane association of Csp, it is essential for initial membrane targeting.

INTRODUCTION

Cysteine-string proteins (Csps) are synaptic-vesicle and secretory-granule proteins which have been shown to function in the Ca²⁺-dependent release of neurotransmitters from nerve endings [1,2]. In the absence of Csp expression in *Drosophila*, the flies either die at a larval stage or, in the case of the few that reach adulthood, die prematurely [1]. Csps are thought to regulate presynaptic Ca²⁺-channel activity [3], but their widespread distribution [4,5] implies that they have an additional, more general, function in secretion. Indeed, recent work has suggested that Csp may co-operate with 70 kDa heat-shock cognate protein (Hsc70) to function as chaperone machinery, ensuring the correct conformational status of one or more secretory-vesicle proteins [6–8].

Csps contain multiple cysteines within their cysteine-string domain, most of which are palmitoylated [9]. The association of Csps with secretory vesicles was initially proposed to be accomplished by the hydrophobic interaction of this palmitoylated cysteine-string domain of Csp with the outer leaflet of the secretory-vesicle membrane [9]. However, more recent work has shown that chemical depalmitoylation of membrane-associated Csp in *Drosophila* does not cause membrane detachment [10], leading to the suggestion that the palmitoylated cysteine-string domain of Csp is not involved in stable membrane association of the protein.

A recent study by Gonzalo and Linder [11] has found that newly synthesized SNAP-25 (synaptosomal-associated protein of 25 kDa) becomes associated with the plasma membrane only once it has been palmitoylated, and compounds which inhibit palmitoylation of SNAP-25 prevent its membrane attachment. However, once membrane-associated, chemical depalmitoylation of SNAP-25 does not result in its displacement from the plasma membrane. The authors concluded, therefore, that palmitoylation of SNAP-25 is required for initial membrane targeting, but that once present at the plasma membrane other domains of the SNAP-25 protein are involved in maintaining a stable membrane association.

We were interested to determine whether palmitoylation of the cysteine-string domain of Csp serves a similar function to that of palmitoylated SNAP-25, and targets mature Csp to secretory-vesicle membranes. To examine this possibility and to resolve the debated question of the role of palmitoylation of Csp in membrane targeting, we have generated a Csp1 mutant protein in which the central core of cysteines in the string domain have been substituted with serines (the 7CS, or seven cysteines to serines, mutant). We have analysed the targeting of this mutant protein to PC12 and HeLa cell membranes in comparison with wild-type Csps, and the results suggest that the cysteine-string domain is indeed required for membrane targeting of Csp.

MATERIALS AND METHODS

Materials

Enhanced chemiluminescence reagents, [^{9,10-³H}]palmitic acid and Amplify fluorographic reagent were purchased from Amersham (Little Chalfont, Bucks, U.K.). QuickChange site-directed mutagenesis kit was obtained from Stratagene (Cambridge, U.K.). Restriction enzymes were from Promega (Madison, WI, U.S.A.). Oligonucleotide primers were synthesized by VH BIO Ltd. (Newcastle-upon-Tyne, U.K.). pcDNA3 plasmid was from Invitrogen (San Diego, CA, U.S.A.). Recombinant His₆-tagged Csp1 protein and Csp polyclonal antiserum were as described previously [4]. Anti-myc epitope, anti-synaptophysin monoclonal antibodies and all other reagents were obtained from Sigma (Poole, Dorset, U.K.).

Generation of 7CS mutant construct

For transfection, myc-Csp1-pcDNA3 and myc-Csp2-pcDNA3 were generated using standard techniques. The myc-Csp1-pcDNA3 construct was used as the template for generating the 7CS mutant by site-directed mutagenesis using the QuickChange system (Stratagene). The sense and antisense primers used were:

Abbreviations used: Csp, cysteine-string protein; PNS, post-nuclear supernatant; SNAP-25, synaptosomal-associated protein of 25 kDa; 7CS, seven cysteines to serines.

¹ To whom correspondence should be addressed (e-mail burgoyne@liverpool.ac.uk).

5'-CTGCTGCTAC(T/A)GT(T/A)GCT(G/C)C(T/A)GCCTC(T/A)GCT(G/C)C(T/A)GCTTCAACTGCTG-3' and 5'-CAG-CAGTTGAAGC(A/T)G(C/G)AGC(A/T)GAGGC(A/T)G-(C/G)AGC(A/T)AC(A/T)GTAGCAGCAG-3', respectively. The nucleotides in brackets indicate the bases that were changed to produce amino acid substitutions. The base substitutions also introduced an additional *DdeI* restriction site into mutant constructs and this allowed simple screening of colonies for mutant plasmids. In addition, the DNA was sequenced in both directions to confirm the fidelity of the mutant construct (Oswell, Southampton, Hants, U.K.).

Cell culture and transfection

PC12 cells were cultured in suspension in RPMI 1640 containing 10% (v/v) horse serum, 5% (v/v) fetal-calf serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂/95% air. Cells were transfected with 10 µg of plasmid DNA using a ProgenitorII PG200 electroporator (Hoefer, San Francisco, CA, U.S.A.) at 1080 µF, 260 V, and with three discharges per sample in a 0.4 cm cuvette. Transfection was performed on 5 × 10⁶ freshly trypsinized cells in 1 ml of culture medium. The transfected cells were maintained in culture for 3 days prior to fractionation analysis. HeLa cells were cultured in Dulbecco's modified Eagle's medium containing 1% (w/v) non-essential amino acids, 10% (v/v) fetal-calf serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin. Transfection of HeLa cells was by the calcium phosphate method (Promega).

Cell fractionation and immunofluorescence analysis

Transfected cells were washed in PBS, resuspended in 300 µl of cell-lysis buffer (5 mM Hepes/5 mM EDTA, pH 7.3) and subjected to a freeze-thaw cycle. The lysed cells were centrifuged at 2000 g for 5 min to pellet nuclei and cell debris. The post-nuclear supernatant (PNS) was centrifuged at 16000 g for 25 min to pellet cell membranes. The supernatant from this centrifugation step (cytosol fraction) was precipitated with an equal volume of methanol. The cytosolic and membrane fractions were then resuspended in an equal volume of SDS dissociation buffer, separated by SDS/PAGE on 12.5% polyacrylamide gels and transferred to nitrocellulose for immunoblotting analysis using rabbit polyclonal anti-Csp [4] or mouse monoclonal anti-myc epitope antibodies. For immunofluorescence analysis, cells were fixed in 4% (v/v) formaldehyde in PBS, washed twice in PBS and incubated for 30 min in PBTA (0.1% (w/v) Triton X-100/0.3% (v/v) BSA in PBS). After this, the cells were incubated for 60 min with the anti-myc antibody (1:400) and washed three times in PBTA. The cells were then incubated with biotinylated anti-mouse IgG (1:100) for 60 min followed by streptavidin/Texas Red (1:50) for 30 min.

Chemical treatment of cell membranes

PC12 cell or HeLa membranes were incubated with 1 M hydroxylamine (pH 7) or 1 M Tris (pH 7) for 20 h at room temperature. The membranes were then pelleted by centrifugation at 16000 g for 25 min and the membrane and soluble fractions examined by immunoblotting. To analyse the effect of hydroxylamine treatment on the distribution of the 7CS mutant protein, transfected cells were resuspended in 1 M hydroxylamine and subjected to a freeze-thaw cycle. The PNS was recovered by centrifugation at 2000 g for 5 min and left at room temperature for 20 h. The PNS was then centrifuged at 16000 g for 25 min and the membrane and soluble fractions examined by immunoblotting.

[³H]Palmitate labelling

Wild-type PC12 cells or PC12 cells stably over-expressing Csp1 [12] were plated at a density of 3 × 10⁶ on collagen-coated 60 mm dishes. After 2 days in culture, the cells were incubated at 37 °C in RPMI for 30 min, and then in RPMI containing 1 mCi/ml [³H]palmitic acid for 3 h. The labelled cells were washed three times in PBS and the membrane fractions prepared. Hydroxylamine treatment was performed at room temperature for 40 h. The membranes were resuspended in dissociation buffer, separated by SDS/PAGE, stained with Coomassie Brilliant Blue, destained and soaked in Amplify (Amersham) to detect radio-labelled polypeptides by fluorography after a 2 week exposure.

RESULTS

Depalmitoylation does not displace Csp from PC12 cell membranes

PC12 cells express two Csp isoforms; Csp1, which is expressed abundantly and associated predominantly with dense-core secretory granules [12], and Csp2, which is expressed only at very low levels [4]. Csp2 is identical to Csp1 but lacks the C-terminal 31 amino acids. The antiserum used in this study was approximately 100 times more sensitive in detection of Csp1 than Csp2, based on detection of recombinant proteins (L. H. Chamberlain and R. D. Burgoyne, unpublished work) and therefore only detects the Csp1 isoform in PC12 cells. Figure 1(a) shows that Csp1 is almost exclusively associated with the membrane fraction of PC12 cells, as described previously [12]. Incubation of the PC12 cell membranes with 1 M Tris does not affect the mass or distribution of Csp1; however, hydroxylamine treatment results in a molecular-mass shift of Csp1, consistent with depalmitoylation (Figure 1a), as reported for *Torpedo* and *Drosophila* Csp [9,10]. This depalmitoylated Csp1 does not dissociate from PC12 cell membranes but migrates similarly to recombinant Csp1, implying that hydroxylamine treatment causes complete or, at least, extensive depalmitoylation. This idea was confirmed by [³H]palmitate labelling of PC12 cells stably over-expressing Csp1 [12]. Immunoblotting analysis shows that the over-expressing clone has a significant increase in Csp1 content compared with control cells (Figure 1b). Consistent with this, an additional major palmitoylated protein, at the size expected for Csp1, is detected in clone C1 following [³H]palmitate labelling, but not in control cells (Figure 1b). Figure 1(b) shows that no radiolabelled proteins are detected after hydroxylamine treatment of the C1 membranes, confirming that hydroxylamine causes complete depalmitoylation of Csp1 and other proteins. Thus, the palmitic acid residues of Csp are not essential for stable membrane association in PC12 cells.

Mutation of the cysteine-string domain of Csp prevents membrane targeting

As depalmitoylation of cellular Csp1 does not affect its membrane association, it is possible that this post-translational modification is required for some other function of Csp. Alternatively, the palmitoylated cysteine-string domain may be essential for initial membrane targeting of Csp. Figure 2(a) is a schematic diagram of mammalian Csp showing the amino acids present in the cysteine-string domain. This cysteine-rich region consists of 14 cysteine residues in a stretch of 24 amino acids and includes a central region in which 7 of 8 amino acids are cysteines (see shaded box on Figure 2d). To assess the role of Csp palmitoylation in membrane targeting, we used a single round of site-directed mutagenesis to substitute the seven cysteine residues in the central region of the cysteine string of Csp1 with serines. Although

Figure 1 Chemically depalmitoylated Csp1 in PC12 cells remains membrane-associated

(a) PC12 cells were homogenized and the PNS was then centrifuged at 16000 *g* to separate the cytosolic (C) and membrane (M) fractions. PC12 membranes were incubated in either 1 M Tris or 1 M hydroxylamine (HA), pH 7.0, for 20 h at room temperature and separated into soluble (S) and pellet (P) fractions by centrifugation at 16000 *g* for 25 min. The samples were analysed for endogenous Csp1 content by immunoblotting with anti-Csp IgG (1:1000) and compared with 5 ng of recombinant Csp1. The blot shows the position of migration of the Csp monomer. (b) Wild-type PC12 cells (WT) and PC12 cells stably over-expressing Csp1 (C1) were incubated with [³H]palmitic acid and the membrane fractions prepared from the cells. Immunoblotting of the samples with anti-Csp1 IgG (1:1000) demonstrates the significant increase in Csp1 expression (both monomer and dimer forms, arrowheads on left-hand side) in C1 cells compared with wild-type cells, and shows that HA treatment of C1 membranes causes a molecular-mass shift in Csp1. Fluorography of the samples to reveal [³H]palmitic acid labelling showed that the C1 clone had a prominent palmitoylated protein (arrow on right-hand side), corresponding in size to Csp1, which was not detected in wild-type cells. No radiolabelled protein corresponding in size to Csp1 or any other proteins was detected in HA-treated C1 membranes.

the efficiency of the mutagenesis was very low (approximately 0.2% as efficient as a similar 2 bp substitution), a mutant plasmid construct was obtained and named 7CS.

PC12 cells were transfected with myc-tagged constructs of Csp1, Csp2 and the 7CS mutant, and the cells were fractionated to examine the extent of membrane association of the expressed proteins. Figure 2(b) shows that, whereas monomer and dimer forms of Csp1 are detected almost exclusively within the membrane fraction, the 7CS mutant protein, which runs predominantly as a 29 kDa monomer, is found only in the cytosolic fraction. Thus, mutation of the cysteine-string domain of Csp1 prevents its membrane targeting. The 7CS protein migrates in a similar position to non-palmitoylated recombinant Csp1 on SDS gels, consistent with the absence or low levels of palmitoylation.

Csp2 is expressed at very low levels in cells [4] and the lack of availability of antisera specific for this truncated isoform has hindered analysis of its distribution. Figure 2(b) shows that myc-tagged Csp2 transfected into PC12 cells also associates exclusively

Figure 2 Unlike wild-type Csp1 and Csp2, the 7CS mutant protein is not targeted to PC12 cell membranes

(a) Schematic diagram of Csp1 and the cysteine residues mutated to serines in the 7CS mutant. The positions of the J domain [4–6] and the cysteine string are indicated. The amino acids in the cysteine-string region are shown, with the central cysteine-rich region highlighted. The seven boxed cysteines were mutated to serines in the 7CS mutant. (b) PC12 cells were transfected with 10 μ g of myc-tagged wild-type Csp1, Csp2 or 7CS mutant Csp1 plasmid DNA. After 3 days the cells were lysed and the PNS centrifuged at 16000 *g* for 25 min to separate cytosolic (C) and membrane (M) fractions. The fractions were then analysed for Csp content by immunoblotting with anti-myc monoclonal antibody (1:1000). The Csp2 immunoblot was subjected to a longer exposure to reveal the migration of the Csp2 monomer at 29 kDa. (c) PC12 cells were transfected with 10 μ g of myc-tagged 7CS plasmid DNA. After 3 days the cells were subjected to a freeze–thaw cycle in lysis buffer (control) or in 1 M hydroxylamine, pH 7.0 (HA). The PNS from the HA treatment was incubated at room temperature for 20 h and then centrifuged to yield supernatant (S) and pellet (P) fractions. The fractions were probed for 7CS content by immunoblotting with anti-myc antibody and compared with the cytosol fraction of untreated transfected cells (control).

with membranes, demonstrating that the C-terminal 31 amino acids of Csp1 are not required for membrane association. From Figure 2(b) it can also be seen that Csp2 has a high tendency to dimerize and this observation may aid future analysis of Csp2 distribution.

The effect of hydroxylamine treatment on the 7CS mutant protein was examined. Hydroxylamine treatment does not affect the migration of 7CS on SDS gels (Figure 2c), suggesting that the mutant protein is not or is very minimally palmitoylated. In addition, hydroxylamine treatment does not cause membrane association of the 7CS protein. This finding is important as it

Figure 3 Csp1, but not 7CS mutant Csp1, expressed in HeLa cells is palmitoylated, membrane-bound and hydroxylamine-resistant

(a) Immunofluorescence analysis of Csp1 distribution in transfected HeLa cells. Cells were incubated with anti-myc antibody (1:400) and then with biotinylated anti-mouse IgG and streptavidin/Texas Red. The scale bar represents 20 μm. (b) Transfected cells were lysed by freeze-thawing and the PNS centrifuged at 16000 *g* for 25 min to separate cytosol (C) and membrane (M) fractions. The membrane fractions were treated with 1 M hydroxylamine (HA), pH 7.0, for 20 h and the soluble (S) and pellet (P) fractions separated by centrifugation at 16000 *g* for 25 min. The fractions were probed with anti-myc antibody.

suggests that continued membrane association of depalmitoylated Csp is not an artefactual consequence of treatment with hydroxylamine.

Csp1 expressed in HeLa cells is also targeted to membranes and remains associated with membranes following hydroxylamine treatment

To further characterize the targeting of Csp1 to cellular membranes, Csp1 was transfected into HeLa cells, a cell line that lacks a regulated secretory pathway. Immunofluorescence analysis of transfected cells (Figure 3a) shows that Csp1 is associated with cellular organelles in this cell type, but is not evident at the plasma membrane. Figure 3(b) demonstrates that the majority of Csp1 is membrane-associated in HeLa cells and runs as expected

for palmitoylated Csp1. A soluble pool of Csp1 co-migrates with depalmitoylated Csp1. Upon hydroxylamine treatment of the HeLa cell membranes, approximately 60 % of Csp1 remains membrane-bound, suggesting that Csp1 can stably associate with membranes other than on secretory vesicles. As seen for PC12 cells, 7CS is completely soluble when transfected into HeLa cells and co-migrates with depalmitoylated Csp1 (Figure 3b).

DISCUSSION

A recent study reported that *Drosophila* Csp remains associated with membranes despite the chemical removal of palmitic acid residues from the cysteine-string domain [10]. One speculation is that the high degree of palmitoylation of Csp may play a central role in exocytotic membrane fusion [13] rather than being involved in membrane attachment. In this study we also found that depalmitoylation of Csp1 by hydroxylamine treatment does not dissociate it from PC12 or HeLa cell membranes, supporting the work of Van de Goor and Kelly [10] on *Drosophila* Csp. In addition, we were able to demonstrate, using [³H]palmitate labelling, that hydroxylamine treatment causes complete depalmitoylation of Csp1 in PC12 cell membranes.

SNAP-25 is a plasma membrane protein which is involved in regulated exocytosis [14,15]. Palmitoylation of SNAP-25 is essential for its initial targeting to, but not association with, the plasma membrane [11,16]. This finding, in addition to previous work, which had implied a correlation between palmitoylation and membrane association of Csp [9], suggested that Csp may need to be modified by fatty acylation to be targeted to membranes. To test this possibility, we constructed a mutant (7CS) protein in which the central core of cysteine residues in the cysteine-string domain was substituted with serines. We chose to mutate the cysteine residues to serines as both amino acids are small uncharged amino acids that have similar overall structures and, therefore, this substitution was likely to have limited structural effects on the protein. Other studies have employed this same amino acid substitution when examining the role of protein palmitoylation in membrane targeting and attachment [17,18].

The 7CS mutant protein did not appear to be extensively palmitoylated when transfected into PC12 cells, as its migration on SDS gels was not noticeably affected by hydroxylamine treatment, as is the case for nonpost-translationally modified protein. This suggests that it may only be the central core of the cysteine-string domain that is palmitoylated, and not the additional flanking cysteine residues. On the other hand, it is possible that the central core of cysteines must be present for palmitoylation of the other cysteines in the string domain to occur. This idea is consistent with SNAP-25 palmitoylation, which is abolished when two of the four palmitoylated cysteine residues are mutated [19]. When the 7CS mutant was transfected into PC12 or HeLa cells it remained entirely cytosolic in contrast with wild-type Csp1 and Csp2, which were present exclusively within the membrane fraction. Analysis of Csp1 distribution in transfected HeLa cells showed that there were two forms of Csp1 in these cells: a membrane-bound palmitoylated pool and a soluble, apparently non-palmitoylated, pool, consistent with the idea that only fully palmitoylated Csp1 becomes membrane-associated. These observations suggest that a palmitoylated cysteine-string domain is required for the targeting of Csp1 to PC12 and HeLa cell membranes.

Whereas the cysteine-string domain is involved in membrane attachment, other regions of the Csp protein must presumably interact with the secretory-vesicle membrane. As Csp2 associates

with membranes to the same extent as Csp1, it is unlikely that the C-terminal 31 amino acids of Csp1 are essential for membrane attachment. Treatment with hydroxylamine did not cause the 7CS mutant to become associated with membranes, implying that continued membrane association of depalmitoylated wild-type Csp is not caused by some artefactual membrane association as a result of hydroxylamine treatment. This strengthens the view that other physiological interactions preserve Csp membrane attachment in the absence of palmitoylation following initial membrane targeting.

The amino acid sequence of Csp contains only one predicted transmembrane domain [20] and the non-palmitoylated protein is highly charged. Therefore, the association of Csp with secretory-vesicle membranes in the absence of palmitoylation may involve an electrostatic- or hydrogen-bonding-dependent interaction with an integral membrane protein(s), and this is consistent with the finding that sodium carbonate releases a portion of Csp from *Drosophila* membranes [10]. These interactions may also serve as important targeting signals for ensuring the exclusive sorting of Csp to secretory-vesicle membranes [21–23]. However, interestingly, a significant portion of Csp1 expressed in HeLa cells was not released from the membranes by hydroxylamine treatment. This suggests that Csp1 can remain associated with cell membranes other than regulated secretory vesicles in the absence of palmitoylation, and argues against an interaction of Csp1 with only a specific regulated secretory-vesicle component. One possibility is an interaction with a vesicle-associated membrane protein (VAMP) homologue, since Csp has been found to interact with neuronal VAMP based on immunoprecipitation from brain extracts [24].

The work presented here resolves the disputed role of the palmitoylated cysteine string in Csp function [9,10] and suggests that it is essential for membrane targeting of the protein. Relatively little is known about the mechanisms by which secretory-vesicle membrane proteins are targeted specifically or the sequence determinants involved. Further work will now be required to determine the mechanism by which Csp is selectively sorted to secretory vesicles rather than to other cellular membranes.

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