Association of *Drosophila* cysteine string proteins with membranes

Jana van de Goor, Regis B. Kelly*

Hormone Research Institute and Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143-0534, USA

Received 21 December 1995

Abstract Cysteine string proteins are putative synaptic vesicle proteins that lack a transmembrane domain. Our analysis shows that *Drosophila* cysteine string proteins are extensively modified by hydroxylamine-sensitive fatty acylation. This modification could be responsible for association of csp's with membranes. Extensive deacylation of Dcsp's by a 20 h incubation in 1 M hydroxylamine, pH 7.0, or methanolic KOH produces a protein of 6–7 kDa lower mass than untreated Dcsp's. Surprisingly, the hydroxylamine treatment does not cause release of Dcsp's from membranes. On the other hand, alkaline stripping of membranes isolated from *Drosophila* brain by 0.1 M sodium carbonate, pH 11.5, causes a significant release of Dcsp's from membranes into the cytosol. These results indicate that fatty acylation may not form the main anchor of Dcsp's in membranes. Taking advantage of the endocytotic block in the *Drosophila* mutant *shibire*^{ts1}, we analyzed the acylation states of Dcsp's in two stages during synaptic vesicle recycling and found no evidence for an acylation/deacylation cycle of Dcsp's in the brain nerve terminals.

Key words: Cysteine string proteins; Post-translational modification; *Drosophila*; Fatty acylation; Synaptic vesicle

1. Introduction

Cysteine string proteins were first identified in a screen of a cDNA library with a monoclonal antibody specific for the *Drosophila* adult nervous system. Sequencing revealed a striking primary sequence containing a contiguous string of 11 cysteine residues. The *Torpedo* homologue of fly csp's was discovered in a search for components or modulators of presynaptic calcium channels [1]. It was shown that antisense RNA of the *Torpedo* csp inhibited the expression of ω-conotoxin-sensitive, dihydropyridine-resistant calcium channels when frog oocytes were injected with *Torpedo* electric lobe mRNA. This result suggested that csp's may be an essential subunit or a modulator of presynaptic calcium channels and so should be localized to presynaptic membrane. Instead, csp's were found to copurify with synaptic vesicles in both *Torpedo* [2] and *Drosophila* [3]. Although the function of csp's is still not understood, their importance in the nervous system becomes obvious from an analysis of null alleles in *Drosophila* [4]. These mutants exhibit a semilethal embryonic phenotype. Adult escapers show a temperature-sensitive block in synaptic transmission, followed by paralysis and premature death. The mutant electroretinogram reveals a loss of 'on' and 'off' transients, suggesting a defect in synaptic transmission. These experiments demonstrate that the csp gene is important for optimal synaptic activity but is obviously not indispensable since some flies can survive its complete deletion.

Although csp's do not have a transmembrane domain they were found to be predominantly but not exclusively associated with membranes [5,6]. Antiserum raised against the *Torpedo* csp recognized both a 27 kDa in vitro translation product and a 34 kDa species in *Torpedo* electric organ [5]. The increased molecular mass in electric organ suggested that csp may be posttranslationally modified. Injection of in vitro translated csp into *Xenopus* oocytes revealed a conversion of a soluble 27 kDa csp into a larger molecular weight form which was entirely membrane-associated [6]. Analysis showed that the principle posttranslational modification of *Torpedo* and rat csp's is fatty acylation [6,7] and this modification was predicted to be responsible for association of csp's with membranes.

In this study we present results from a biochemical characterization of *Drosophila* csp's. We show that there are differences in subcellular distribution and membrane association of *Drosophila* csp's compared to the *Torpedo* homologue. We were interested in whether the *Drosophila* homologue is fatty acylated and if this modification is required for membrane association. Our results suggest that fatty acyl groups do not form the main anchor of Dcsp's in membranes since a complete removal of this modification does not dissociate Dcsp's from membranes. In addition we analyzed the acylation states of Dcsp's in two distinct stages of synaptic vesicle recycling, when Dcsp's are on synaptic vesicles and after massive fusion of these vesicles with the plasma membrane in *shibire* flies.

2. Materials and methods

2.1. Experimental animals

The experimental animals were the wild-type strain of *Drosophila melanogaster*, and the temperature-sensitive mutant, *shibire*^{ts1}. Flies were cultured in standard sugar/agar-containing medium in bottles at 19°C. To deplete nerve terminals of synaptic vesicles, *shibire* flies were incubated at 32°C for 15 min and the paralyzed flies were rapidly frozen in liquid nitrogen. Flies (10–15 g) were decapitated and heads were collected using a sieve. Frozen heads were ground in a mortar and pestle. All subsequent steps were done at 4°C. The resulting powder was resuspended in 1 ml of buffer A (150 mM NaCl, 10 mM HEPES, pH 7.4, 1 mM EGTA, 0.1 mM $MgCl_2$) and homogenized by 20 strokes in a glass–glass homogenizer. Post-nuclear supernatant was prepared by a centrifugaton at 1,000×*g* for 10 min.

2.2. Antibodies and immunoblotting

A monoclonal antibody to Dcsp's, mAb 49, was obtained from Drs. E. Buchner and K. Zinsmaier [8]. Secondary antibodies (HRP-conjugated, affinity-purified goat anti-mouse were purchased from Cappel. Antibody SVP-38 (Sigma) recognizing rat synaptophysin was used in Western blot analyses of subcellular fractionation of PC12 cells.

Samples were run on 12% SDS-PAGE, transferred to Immobilon-P (Millipore) using a semi-dry blotter (E and K) and analyzed by Western blotting. Prior to incubation with specific antibodies, blots were blocked in PBS/0.05% Tween 20/1% BSA. All primary and secondary

*Corresponding author. Fax: (1) (415) 731-3612.

Abbreviations: Dcsp's, *Drosophila* cysteine string proteins;; T-csp, Torpedo cysteine string protein.

SSDI S0014-5793(96)00026-9

antibody incubations were performed in blocking buffer. Bands were visualized using the ECL system (Amersham).

2.3. Biochemistry of Drosophila csp's

For deacylation of Dcsp's, heads of wild-type *Drosophila* were collected and homogenized as described above. A post-nuclear supernatant was obtained by a 10 min centrifugation at 1,000×*g* at 4°C. Aliquots of the supernatant were treated with 1 M hydroxylamine, pH 7.0 for 2 and 20 h, or with 0.1 M methanolic KOH for 2 h at room temperature. As control an aliquot was incubated with 1 M Tris, pH 7.0, for 20 h. For membrane association experiments aliquots of post-nuclear supernatant were subjected to a 125,000×*g* centrifugation for 75 min to separate cellular membranes from soluble proteins. Pellets of cellular membranes were resuspended in buffer A or 0.1 M sodium carbonate, pH 11.5 or 0.5 M NaCl and incubated for 1 h at 4°C, or resuspended in 1 M hydroxylamine and incubated for 20 h. Incubations were followed by an additional centrifugation to separate membranes and soluble proteins. All pellets were finally dissolved in 200 µl of 1×sample buffer and equal volumes of supernatants and dissolved pellets were run on 12% SDS-PAGE followed by a Western blot analysis using Mab49 antibody.

2.4. Expression of Dcsp in PC12 cells

Dcsp cDNA (pK49-4b) was a gift of Dr. K. Zinsmaier. The csp coding sequence was cut out with *KpnI*/*XbaI* and cloned into a mammalian expression vector pCB6. PC12 cells were grown in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose (DME H-21) supplemented with 10% horse serum, 5% fetal bovine serum, 10 U/ml penicillin and 100 µl/ml streptomycin in a humidified incubator with 10% CO_2. Cells were transiently transfected with the pCB6-Dcsp construct by electroporation. Rapidly dividing cells were washed, pelleted and resuspended in electroporation buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose, 20 mM HEPES, pH 7.05) at ~4×10^7 cells/ml. 50 µg of pCB6-Dcsp was mixed with 0.8 ml of cell suspension. The mixture was incubated in electroporation cuvettes at room temperature for 5 min, pulsed at 230 V, 500 µF and incubated for an additional 20 min at 37°C before plating the cells onto a tissue culture dish. After 24 h the medium was changed to a fresh one containing 6 mM sodium butyrate to enhance expression of the recombinant plasmid. Transfected cells (48 h after electroporation) were harvested and homogenized in buffer A by 7 passes through a ball-bearing cell cracker (European Molecular Biology Laboratory, 12 µm clearance) on ice. A post-nuclear supernatant was prepared by centrifugation at 1,000×*g* for 10 min.

2.5. Subcellular fractionation

Post-nuclear supenatants from PC12 cells and *Drosophila* heads were layered on a 5–25% glycerol gradient in buffer A over a sucrose pad and the gradient was centrifuged for 1 h at 48,000 rpm in a SW55 Ti rotor (Beckman). Fractions were collected from the bottom and assayed by Western blot analysis.

3. Results

3.1. Drosophila csp's are fatty acylated

A postnuclear supernatant from *Drosophila* heads was treated with a deacylating reagent, 1 M hydroxylamine, pH 7.0, which is known to cleave fatty acyl thio-esters of proteins [9,10]. Over a two-hour time course, deacylation resulted in a maximal shift of approximately 6–7 kDa with several intermediates between the fully modified and fully deacylated forms (Fig. 1), suggesting that several residues are acylated. The sensitivity to hydrolysis by neutral hydroxylamine indicates that Dcsp's incorporate fatty acids predominantly by a thioester linkage to cysteine residues. Only after prolonged incubation (20 h) at room temperature was most of the immunoreactivity present in two immunoreactive bands of lower molecular weight, corresponding to the two Dcsp isoforms recognized by the ab49 antibody [7]. The same decrease in mass was observed when the sample was treated with methanolic 0.1 M KOH. To rule out that these lower molecular weight bands result from proteolytic degradation, an aliquot of postnuclear supernatant was incubated with 1 M Tris pH 7.0 under the same conditions. No degradation products were observed.

3.2. Drosophila csp's can be displaced from membranes by carbonate stripping

Our previous study showed that *Drosophila* csp's are present on synaptic vesicles as well as on plasma membrane and recycle together with other synaptic vesicle markers between both types of membranes [3]. Since csp's lack a transmembrane domain their association with membranes is undefined. In homogenates from *Drosophila* heads, all Dcsp immunoreactivity was associated with membranes (Fig. 2). We did not detect any soluble pool of Dcsp's, unlike that reported for csp extracted from *Torpedo* electric organ [5,6]. When isolated membranes were incubated in 100 mM Na_2CO_3, pH 11.5, a condition that is known to release peripheral proteins from membranes, a substantial fraction of the Dcsp immunoreactivity was displaced into the supernatant. No immunoreactivity was detected in the supernatant following a low salt (buffer A) or high salt (0.5 M NaCl) wash. This release from membranes was not accompanied by any change in Dcsp mobility indicating that high pH did not hydrolyse the thioester bonds between the fatty acids and the cysteine residues. These results suggest that Dcsp's are peripheral membrane proteins and can be released with a full complement of fatty acids by carbonate stripping.

3.3. Deacylation does not cause the release of Dcsp's from membranes

Isolated membranes from *Drosophila* brains were treated for an extensive period of time (20 h at room temperature)

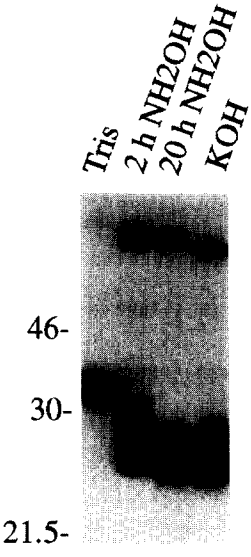

Fig. 1. *Drosophila* cysteine string proteins are fatty acylated. A post-nuclear supernatant of *Drosophila* brain (1,000×*g*, 10 min) was treated with deacylating agents 1 M hydroxylamine, pH 7.0, and with 0.1 M methanolic KOH. The 2 h incubation with hydroxylamine resulted in numerous deacylation intermediates while after the 20 h incubation most immunoreactivity was concentrated in a band of 6–7 kDa lower mass than untreated sample. The mobility of this band was identical to KOH-treated sample. Incubation in 1 M Tris, pH 7.0, for 20 h was used as control. Numbers on the left indicate molecular weight markers in kDa.

with 1 M hydroxylamine. The immunoreactive product showed a 6–7 kDa reduction in mass when compared to untreated sample and so was deacylated (Fig. 3). No deacylation intermediates were observed and the migration on an SDS-PAGE was identical to a methanolic-KOH treated sample. This suggests that all acyl groups were removed by this treatment. Surprisingly, even after the prolonged deacylation treatment and a subsequent 0.5 M NaCl wash, no immunoreactivity was detected in the supernatant. This result is consistent with our observation that carbonate stripping releases Dcsp's from membranes and implicates interactions that do not involve cysteine-linked fatty acylation in anchoring Dcsp's on the membranes.

In addition to a shift to lower molecular weight upon deacylation, some Dcsp immunoreactivity was consistently recovered in a band of approximately twice the mass of Dcsp monomers (Fig. 3). We do not know if this reflects formation of a dimer or association of Dcsp's with another protein of a similar molecular weight.

3.4. Unmodified Dcsp's do not associate with membranes

It has been demonstrated that post-translationally modified *Torpedo* csp is membrane-associated while unmodified Tcsp is soluble [6]. In an attempt to examine how membrane association of *Drosophila* csp's take place we transiently expressed Dcsp cDNA under a CMV promoter in the neuroendocrine cell line, PC12 and performed a subcellular fractionation by glycerol velocity sedimentation (Fig. 4). Transfected *Drosophila* csp was not post-translationally modified, as evidenced by the lower molecular weight on SDS-PAGE, and did not associate with membranes. All Dcsp was soluble and sedimented at the top of the gradient (Fig. 4, middle), suggesting that, as in *Torpedo*, Dcsp requires a post-translational modification to associate with membranes. Subcellular distribution of post-translationally modified Dcsp's in *Drosophila* brain shows targetting to synaptic vesicles and large membranes (Fig. 4, top). The position of synaptic vesicles and plasma membrane in fractionation of PC12 cells by glycerol velocity sedimentation is shown using synaptophysin as a marker (Fig. 4, bottom). The anti-Dcsp antibody did not crossreact with endogenous rat csp in PC12 cells (data not shown).

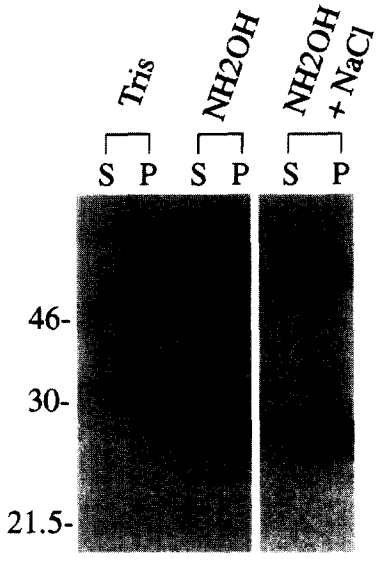

Fig. 3. Deacylation by hydroxylamine does not displace Dcsp's from membranes. A post-nuclear supernatant from *Drosophila* brain was centrifuged at 125,000×*g* for 75 min. The membrane pellet was treated with 1 M hydroxylamine, pH 7.0, for 20 h at room temperature. The sample was subsequently centrifuged at 125,000×*g* for 75 min to separate cellular membranes (P) from soluble proteins (S). Although deacylation seems to be complete and without the presence of deacylation intermediates, all Dcsp's stayed membrane-associated. Even a subsequent incubation in 0.5 M NaCl did not displace Dcsp's from membranes. As control, membrane pellet was incubated in 1 M Tris, pH 7.0.

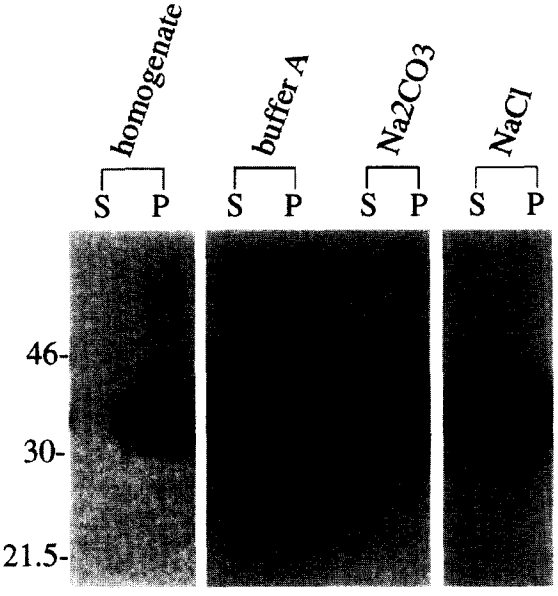

Fig. 2. *Drosophila* csp's can be displaced from the membranes to the supernatant by a carbonate but not by a high salt incubation. A post-nuclear supernatant from *Drosophila* brain homogenate was centrifuged at 125,000×*g* for 75 min to separate membrane pellets (P) from soluble proteins (S). Dcsp's were found to be present exclusively in the membrane pellet and were absent from the cytosol. Membrane pellets were resuspended in low salt (buffer A), high salt (0.5 M NaCl) or 0.1 M sodium carbonate, pH 11.5 and incubated for 1 h at 4°C. Membranes and soluble proteins were separated again by centrifugation and analyzed by a Western blot analysis. From the reagents tested, only sodium carbonate displaced a substantial portion of Dcsp's from membranes into the supernatant. Note that carbonate treatment did not cause hydrolysis of the thioester bonds since the mobility of carbonate-treated Dcsp's is identical to the untreated ones.

3.5. Dcsp's do not change acylation state during synaptic vesicle recycling

Several fatty acylated proteins have been shown to undergo cycles of acylation/deacylation in the cell. To learn whether Dcsp's change acylation state during recycling of synaptic vesicles in the nerve terminal, we compared the electrophoretic mobility of Dcsp's in *shibire* flies at permissive and restrictive temperatures. At restrictive temperatures the recycling of synaptic vesicles in *shibire*^{ts1} mutant becomes blocked at an early stage of endocytotic retrieval of vesicles from the plasma membrane. We have shown previously that all synaptic vesicles are depleted from the nerve terminals during exposure of *shibire* flies to elevated temperatures [3]. The synaptic vesicle antigens, synaptotagmin, synaptobrevin and Dcsp's were dramatically absent from synaptic vesicle fractions in *shibire* flies at restrictive temperatures. Instead, they were recovered in the plasma membrane containing fractions. We can thus analyze Dcsp acylation in two distinct stages of synaptic vesicle recy-

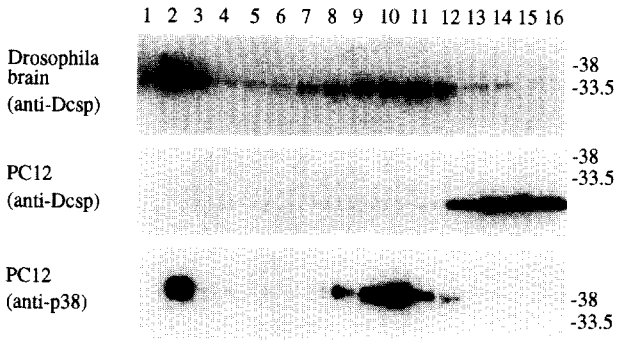

Fig. 4. Subcellular localization of Dcsp in neuroendocrine cell line PC12 analyzed by a glycerol velocity sedimentation. Post-nuclear supernatants from *Drosophila* brain and transfected PC12 cells were subjected to fractionation by a glycerol velocity sedimentation and gradient fractions were analysed by Western blot analysis. Dcsp's in *Drosophila* brain are localized to large membranes (fraction 1–3) and to synaptic vesicles (fraction 9–12). Expression of Dcsp in PC12 cells shows a lack of post-translational modifications and localization to the cytosol (fraction 13–16). An established synaptic vesicle marker in PC12 cells, synaptophysin (p38), shows the position of large membranes (fraction 2) and synaptic vesicles (fraction 8–12) in subcellular fractionation of PC12 cells.

cling: in a steady-state, when Dcsp's are associated with synaptic vesicles, and after massive fusion of synaptic vesicles with the plasma membrane. We fractionated post-nuclear supernatants from *shibire* flies incubated at permissive and restrictive temperatures and analyzed fractions containing synaptic vesicles and plasma membrane (Fig. 5). We detected no differences when the electrophoretic mobilities of Dcsp's on synaptic vesicles and after transfer to the plasma membrane were compared. Thus, acylation does not appear to change dramatically during the synaptic vesicle cycle.

4. Discussion

Our previous work showed that *Drosophila* cysteine string proteins are authentic synaptic vesicle proteins. When we analyzed their distribution in homogenates from *Drosophila* heads, we found that all Dcsp's were associated with either synaptic vesicles or large membranes cosedimenting with a plasma membrane marker. Thus, all Dcsp's was found to be membrane-bound. Unlike our findings with the protein Rop (a sec-1 homologue in yeast), none of the Dcsp's was recovered at the top of the gradient as soluble protein [3,11]. When subcellular fractionation was performed in *Torpedo* electric organ a considerable fraction of the Dcsp was recovered as soluble material [5,6]. If the soluble form was a neuronal precursor more of it would be expected in homogenates of brain cells than in homogenates of electric organ, which does not contain neuronal cell bodies.

Cysteine string proteins derive their name from the remarkable string of cysteine residues that lie next to each other in the sequence. *Torpedo* as well as rat csp's have been shown to be extensively fatty acylated on most of the cysteine residues in the string of cysteines [6,8] and these fatty acyl tails were implicated in anchoring csp's in membrane. First we investigated the presence of fatty acylation in the *Drosophila* csp homologues. Both reagents that are known to selectively cleave thioester linked fatty acyl groups, 1 M hydroxylamine, pH 7.0 and methanolic KOH, reduced the mass of Dcsp's by 6–7 kDa. Although we have no direct measure of the number of cysteine residues that are acylated, the large difference in electrophoretic mobility and the presence of numerous partially hydrolyzed intermediates strongly suggests that a large number of the cysteine residues are modified. Estimating the number of fatty acyls from the number of Dcsp bands cannot be done satisfactorily because the anti-Dcsp antibody recognizes more than one Dcsp isoform.

Fatty acylation is a frequent modification whose biological significance remains obscure. There are two major pathways for this covalent modification: cotranslational attachment of 14-carbon fatty acid, myristate, through amide linkage to the N-terminus of some proteins, and an attachment of 16-carbon palmitate or other long-chain fatty acids through ester linkages that take place posttranslationally. Postranslational acylation can be subdivided in early and later postranslational types, the latter one being dynamic and sensitive to extracellular stimuli [12]. Fatty acylation has been implicated in cellular events such as protein–protein and protein–lipid interactions. Unlike myristoylation, palmitoylation of cysteine residues is a reversible modification. Pulse-chase experiments have demonstrated a fast turnover of palmitate group of several proteins, including H-ras and N-Ras [13], the transferrin receptor [14] and neuronal growth cone protein GAP-43 [15]. It is possible that this dynamic modification, like reversible phosphorylation, can regulate a variety of cellular functions. An acylation-deacylation cycle has been implicated in regulated translocation of several proteins between cytosol and membranes. Thus, activation of G protein α-subunit by hormone receptor or by mutation causes rapid depalmitoylation, providing an explanation for translocation from the membrane to the cytoplasm [16]. One of the G protein-coupled receptor kinases, GRK6, also undergoes an acylation cycle associated with stimulus-dependent membrane association

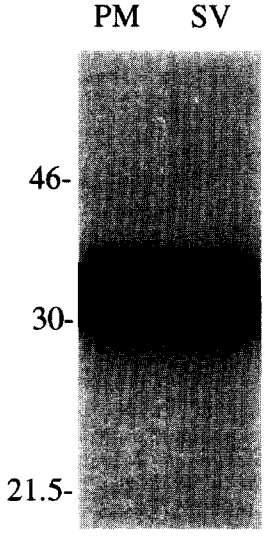

Fig. 5. Dcsp's do not undergo acylation/deacylation cycle during synaptic vesicle recycling in the nerve terminals. Post-nuclear supernatants from heads of *shibire* flies kept at permissive temperature, and after exposure to restrictive temperature were fractionated by glycerol velocity sedimentation. Fractions containing synaptic vesicles (SV) or plasma membrane after massive fusion of vesicles in a *shibire* block (PM) were analyzed by a Western blot analysis. Analysis shows that Dcsp's are fully acylated in both stages of synaptic vesicle recycling.

[17]. Taking advantage of the *shibire* mutant we were able to analyze Dcsp's when they are present on synaptic vesicles and after fusion of vesicles with the plasma membrane. Our results suggest that Dcsp's do not undergo such a translocation nor acylation/deacylation cycle during synaptic vesicle recycling at the nerve terminal since we were unable to detect any soluble pool of Dcsp's in *Drosophila* brain, and Dcsp's on synaptic vesicles and plasma membrane were uniformally acylated.

Alkaline stripping with sodium carbonate, pH 11.5, is often used to distinguish between integral membrane proteins and peripheral ones [18]. When Dcsp's were alkaline-treated a significant amount of the immunoreactivity was displaced from the membrane pellet into the supernatant. This is an unexpected property for fatty acyl-anchored proteins, which should behave like integral membrane proteins. Moreover, our results show that alkaline treatment does not cause hydrolysis of the fatty acyl groups, since we do not detect any change in molecular mass, and yet Dcsp's become soluble. To examine if acylation is responsible for anchoring Dcsp's to *Drosophila* membranes, we examined the distribution of Dcsp immunoreactivity in the membrane and soluble fractions from *Drosophila* brain homogenate after extensive chemical deacylation by hydroxylamine. Surprisingly, all Dcsp stayed membrane-associated. Taken together, these results indicate that fatty acylation may not be the factor involved in Dcsp's association with cellular membranes. A similar conclusion was reached recently for another membrane-associated protein, glutamic acid decarboxylase GAD65 [19]. GAD65 is palmitoylated on two cysteine residues in the NH2-terminal region and this modification was thought to anchor GAD to membranes [20]. Recently it has been demonstrated that deletion of both of these cysteine residues does not interfere with membrane association of GAD which instead requires a short stretch of amino acids for targeting to membranes. It was suggested that palmitoylation of GAD may rather serve to orient or fold the protein at synaptic vesicle membranes. In the case of *Drosophila* cysteine string proteins it is plausible that these fatty acids are free and available for mediating interactions during membrane fusion.

Correlation between a posttranslational modification of csp's and their membrane association has been well documented for *Torpedo* csp. When in vitro translated *Torpedo* csp was injected into oocytes they converted a portion of the 27 kDa soluble form into a higher mass that became exclusively membrane-associated [6]. We transiently expressed *Drosophila* csp in PC12 cells to examine the link between posttranslational modification and membrane association. Transfected Dcsp was not posttranslationally modified, as judged from its electrophoretic mobility. All Dcsp immunoreactivity sedimented with the soluble proteins demonstrating that unmodified Dcsp does not associate with membranes. It is possible that the signals for modifications are not sufficiently conserved between *Drosophila* and rat, and therefore Dcsp was not modified. It is also possible that Dcsp's cannot become membrane-associated in PC12 cells and therefore do not get acylated. A Dcsp homologue does, however, exist in rat and is membrane-associated [8].

Hydroxylamine treatment is known to hydrolyze thioester bonds but leaves other modifications intact. It is therefore possible that modifications other than fatty acylation are involved in anchoring Dcsp's in membranes.

What might be the function of palmitic acid moieties in csp's? Many integral membrane proteins undergo palmitoylation. In these cases we can be confident that membrane association is not the function. Palmitoylation of receptors such as the adrenergic receptor [21] affects desensitization of the response to ligand binding. More pertinent to csp function, however, may be the evidence linking palmitoylation to protein interactions at the plasma membrane. Thus the interaction of the nucleocapsid of *Sindbis* virus with the plasma membrane is defective in palmitoylation-deficient mutants of the E2 membrane glycoprotein [22]. Acylation is also required for influenza virus budding. Finally palmitoylation of the transferrin receptor, induced for example by insulin, inhibited transferrin uptake [23] although this claim is controversial [14]. The possibility that the acyl groups of membrane proteins are involved in membrane traffic received strong support from the in vitro studies of Rothman and his colleagues. Both membrane fusion [24] and membrane budding [25] require the addition of fatty acyl CoA. This is interpreted to mean that acylation of one or more membrane proteins is required for the fission and fusion events. The requirement of acylation in membrane trafficking and the unusually high extent of csp acylation makes it tempting to believe that csp is an important player in the interactions of synaptic vesicles with the plasma membrane.

Acknowledgements: We wish to thank E. Buchner and K. Zinsmaier for donation of antibodies. We acknowledge Leslie Spector for her patient help with the manuscript and members of the Kelly lab for critical reading of the manuscript. The work was funded by NIH Grant NS15927 to R.B.K.

References

[1] Gundersen, C.B. and Umbach, J.A. (1992) Neuron 9, 527–537.
[2] Mastrogiacomo, A., Parsons, S.M., Zampighi, G.A., Jenden, D.J., Umbach, J.A. and Gundersen, C.B. (1994) Science 263, 981–982.
[3] van de Goor, J., Ramaswami, M. and Kelly, R.B. (1995) Proc. Natl. Acad. Sci. USA 92, 5739–5743.
[4] Zinsmaier, K.E., Eberle, K.K., Buchner, E., Walter, N. and Benzer, S. (1994) Science 263, 977–980.
[5] Mastrogiacomo, A., Evans, C.J. and Gundersen, C.B. (1994) J. Neurochem. 62, 873–880.
[6] Gundersen, C.B., Mastrogiacomo, A., Faull, K. and Umbach, J.A. (1994) J. Biol. Chem. 269, 19197–19199.
[7] Mastrogiacomo, A. and Gundersen, C.B. (1995) Mol. Brain. Res. 28, 12–18.
[8] Zinsmaier, K.E., Hofbauer, A., Heimbeck, G., Pflugfelder, G.O., Buchner, S. and Buchner, E. (1990) J. Neurogenet. 7, 15–29.
[9] Magee, A.I., Loyama, A.H., Malfer, C., Wen, D. and Schlesinger, M.J. (1984) Biochim. Biophys. Acta 798, 156–166.
[10] Kaufman, J.F., Krangel, M.S. and Strominger, J.L. (1984) J. Biol. Chem. 259, 7230–7238.
[11] Harrison, S.D., Broadie, K., van de Goor, J. and Rubin, G.M. (1994) Neuron 13, 555–566.
[12] James, G. and Olson, E.N. (1989) J. Biol. Chem. 264, 20998–21006.
[13] Magee, A.I., Gutierrez, L., McKay, I.A., Marshall, C.J. and Hall, A. (1987) EMBO J. 6, 3353–3357.
[14] Jing, S.Q. and Trowbridge, I.S. (1990). J. Biol. Chem. 265, 1555–11559.
[15] Skane, J.H.P. and Virag, H. (1989) J. Cell Biol. 108, 613–624.
[16] Wedegaertner, P.B. and Bourne, H.R. (1994) Cell 77, 1063–1070.
[17] Stoffel, R.H., Randall, R.R., Premont, R.T., Lefkowitz, R.J. and Inglese, J. (1994) J. Biol. Chem. 269, 27791–27794.
[18] Fujiki, Y., Hubbard, A.L., Fowler, S. and Lazarow, P.B. (1982) J. Cell Biol. 93, 979–102.
[19] Shi, Y., Veit, B. and Baekkeskov, S. (1994) J. Cell Biol. 124, 927–34.

[20] Christgau, S., Aanstoot, H.J., Schierbeck, H., Begley, K., Tullin, S., Hejnaes, K. and Baekkeskov, S. (1992). J. Cell Biol. 118, 309–320.
[21] Eason, M.G., Jacinto, M.T., Theiss, C.T. and Liggett, S.B. (1994) Proc. Natl. Acad. Sci. USA 91, 11178–82.
[22] Ivanova, L. and Schlesinger, M.J. (1993) J. Virol. 67, 2546–51.
[23] Alvarez, E., Girones, N. and Davis, R.J. (1990) J. Biol. Chem. 265, 16644–16655.
[24] Pfanner, N., Glick, B.S., Arden, S.R. and Rothman, J.E. (1990) J. Cell Biol. 110, 955–961.
[25] Ostermann, J., Orci, L., Tani, K., Amherdt, M., Ravaztzola, M., Elazar, Z. and Rothman, J.E. (1993) Cell 75, 1015–25.