Intrinsic membrane association of *Drosophila* cysteine string proteins

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Abstract Cysteine string proteins (csps) are highly conserved constituents of vertebrate and invertebrate secretory organelles. Biochemical and immunoprecipitation experiments implied that vertebrate csps were integral membrane proteins that were tethered to the outer leaflet of secretory vesicles via the fatty acyl residues of their extensively acylated cysteine string. Independently, work of others suggested that *Drosophila* csps were peripheral membrane proteins that were anchored to membranes by a mechanism that was independent of the cysteine string and its fatty acyl residues. We extended these investigation and found first that sodium carbonate treatment partially stripped both csps and the integral membrane protein, synaptotagmin, from *Drosophila* membranes. Concomitantly, carbonate released fatty acids into the medium, arguing that it has a mild, solubilizing effect on these membranes. Second, we observed that *Drosophila* csps behaved like integral membrane proteins in Triton X-114 partitioning experiments. Third, we found that when membrane-bound csps were deacylated, they remained membrane bound. Moreover, it appeared that hydrophobic interactions were necessary for this persistent membrane association of csps. Thus, neither reducing conditions, urea, nor chaotropic agents displaced deacylated csps from membranes. Only detergents were effective in solubilizing deacylated csps. Finally, by virtue of the inaccessibility of deacylated csps to thiol alkylation by the membrane-impermeant alkylating reagent, iodoacetic acid, we inferred that it was the cysteine string domain that mediated the membrane association of deacylated csps. Thus, we conclude that under physiological conditions csps are integral membrane proteins of secretory organelles, and that the cysteine string domain plays a vital role in the membrane association of these proteins.

Key words: Cysteine string protein; Membrane protein; Protein fatty acylation; Synaptic vesicle; Nerve terminal

1. Introduction

Cysteine string proteins (csps) were first described as a pair of relatively small proteins (< 250 amino acid residues) derived from alternative splicing of a primary RNA transcript in *Drosophila* [1]. These proteins were also shown to be localized predominantly at synapses in the central and peripheral nervous system of *Drosophila* [1]. Independently, a vertebrate csp was identified [2], and shown to be associated with synaptic vesicles from *Torpedo* electric organ [3]. Csps have since been found to comprise a family of proteins that are highly conserved from nematode worms to man [4,5]. In addition, csps have now been shown to be constituents of a variety of regulated secretory organelles (e.g. adrenal chromaffin granules) besides synaptic vesicles [6–11]. Investigations of csp function using *csp* mutant *Drosophila* have revealed that these proteins are vital for excitation-secretion coupling at larval neuromuscular junctions [12]. However, the precise role of csps in the regulated secretory pathway has not been established [4].

An important aspect of efforts to understand the contribution of csps to regulated secretion is to determine the mechanism by which csps associate with secretory organelles. Among the first experiments addressing this issue were studies of the sedimentation properties, solubility (in alkaline or detergent solutions) and Triton X-114 partitioning behavior of csps in *Torpedo* electric organ [13]. This work led to the conclusion that csps were integral membrane proteins in *Torpedo* electric tissue [13]. Later it was documented that nearly all of the cysteine residues of the cysteine string of csp were fatty acylated [14]. These data, coupled with the observation that antibodies selective for the amino- or carboxyl-terminal domains of csps (these domains flank the cysteine string) immunoprecipitated synaptic vesicles [3] led to the hypothesis that csps were tethered to the P-face of secretory organelles (via the fatty acyl residues of their cysteine string) with the amino- and carboxyl-terminal domains projecting toward the cytosol [3,15].

This initial model of the membrane association of csps emerged from studies using *Torpedo* tissue and it has since been challenged by results from studies of *Drosophila* membranes [16]. Thus, based on carbonate-stripping experiments, van de Goor and Kelly [16] concluded that csps were peripheral membrane proteins. Moreover, because they also observed that csps remained membrane-associated after deacylation by hydroxylamine, these investigators concluded that some other region of csp, besides its fatty acylated cysteine string was important for the membrane association of these proteins. We have extended these investigations with the goal of clarifying the nature of the membrane association of *Drosophila* csps.

2. Materials and methods

2.1. Experimental animals and reagents

Wild-type *Drosophila melanogaster* were the Canton S strain. Antibodies against *Drosophila* csp (Dcsp 1, [17]) and antibodies against synaptotagmin (Dsyt 2, [18]) were kind gifts from Drs. K. Zinsmaier and H. Bellen, respectively. All other reagents were either from Sigma or Fisher except for protein molecular weight standards from Amersham.

2.2. Membrane preparations

Drosophila head membranes were obtained by homogenizing (glass:glass) 10 frozen fly heads per 0.1 ml of solution A (0.1 M NaCl, 0.05 M Tris, 1 mM EDTA, 1 mM EGTA, pH 7.4) and centrifuging for 3 min at $1000\times g$ to remove exoskeleton. Where indicated, this solution was supplemented with DTT (5 mM). The resulting supernatant was sedimented for 1 h at $100\,000\times g$ in an airfuge to yield a membrane pellet that is enriched in immunoreactive csps. All

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experiments employed membranes from 3–10 fly heads unless otherwise indicated.

2.3. Alkaline stripping of membranes using sodium carbonate or CAPS solutions

Drosophila membranes (20–50 μg protein) were suspended by trituration in 0.1 ml of solution A or either 0.1 M sodium carbonate (pH 11.5 at 20°C) or 0.1 M CAPS (3-cyclohexylamino-1-propane sulfonic acid, pH 11.5 at 20°C with NaOH) at 4°C. After 1 h, samples were centrifuged for 1 h at 100 000×*g* at 4°C. The supernatant was removed and the pellet was washed with 50 μl of the appropriate homogenization solution. The supernatant and the wash were pooled, the pH was adjusted with 0.1 volume of 1 M Tris (pH 7.0), and protein was recovered by an organic-solvent extraction procedure [19]. Protein from the supernatant and pellet were suspended in sample buffer [20] with 5% SDS for immunoblot analysis.

2.4. Chemical treatment of Drosophila head membranes

Drosophila head membranes were suspended by trituration in several different solutions to explore the nature of the membrane association of native and deacylated csps. These solutions were prepared in solution A which was supplemented with either 1 M hydroxylamine hydrochloride (pH was adjusted to 7.0–7.2 by addition of about 0.8 M equivalents of NaOH) or 1 M Tris (pH 7.0 with HCl) as a control for the hydroxylamine. To these Tris- or hydroxylamine-containing solutions was then added: (i) 50 mM dithiothreitol (DTT); (ii) 50 mM DTT plus 2.5 M KSCN; (iii) 50 mM DTT, plus 8 M urea; and (iv) 50 mM DTT, plus 0.2% SDS. Membranes from 9–10 fly heads were suspended in 75 μl of each solution and incubated for 16–18 h at 20–22°C. Samples were then diluted with 75 μl of H_2O (which reduces solution density and facilitates sedimentation of membranes) and centrifuged 1 h at 100 000×*g* at 4°C. The supernatant and pellet were recovered for immunoblot analysis exactly as described above. To reduce the extent to which both acylated and deacylated csps form thiol-linked complexes, we included 10 mM DTT in the SDS sample buffer and followed this by alkylating samples for 1–2 h at 21°C with 25 mM iodoacetamide in 50 mM Tris (pH 7.5). All alkylation reactions were done in the dark.

In a second series of experiments, fly head membranes were incubated in 1 M hydroxylamine in solution A (pH 7.2) without or with iodoacetic acid (50 mM) in a final volume of 0.1 ml. After 16 h at 22°C, samples were sedimented (1 h at 100 000×*g* at 4°C) and the supernatant and pellet were recovered independently for immunoblot analysis of csps. These samples were not alkylated prior to immunoblotting.

In the third set of experiments, fly head membranes were prepared under reducing conditions (with 5 mM DTT in the homogenization buffer), and were then incubated in 1 M hydroxylamine in solution A (pH 7.2 with NaOH) without or with iodoacetic acid (50 mM) or iodoacetamide (50 mM). In some samples, SDS (1%) was also present. After a 16 h incubation at 22°C, samples were extracted [19] for immunoblot analysis. Where indicated, DTT (50 mM) was present during the extraction and in the SDS sample buffer.

2.5. Triton X-114 partitioning of hydroxylamine-treated membranes from Drosophila heads

These procedures followed the protocol of Bordier [21] for identifying integral membrane proteins. Two fly heads were homogenized at 4°C in 0.1 ml of solution A with 1% Triton X-114. After solubilization for 1–2 h at 4°C, insoluble material was removed by centrifugation (10 min at 14 000×*g* at 4°C) and the soluble material was layered over a cushion (0.2 ml) of 6% sucrose in solution A with 0.1% Triton X-114. This sample was condensed at 37°C for 5 min and centrifuged (500×*g* for 3 min) to separate the Triton X-114 condensate from the supernatant. Protein was recovered [19] from both the supernatant and the Triton X-114 bead and subjected to immunoblot analysis.

2.6. Fatty acid analysis

Membranes from 20 fly heads were suspended in 0.1 ml of either solution A or 0.1 M sodium carbonate (pH 11.5) and incubated on ice. After 1 h the membranes were sedimented (1 h at 100 000×*g* at 4°C) and the supernatants were removed and mixed with an equal volume of either solution A or carbonate solution so that the salt compositions were equivalent. Then, approximately 0.01 ml of 1 M HEPES (free acid) was added to adjust the pH to 7.0. Each sample then received 750 ng of heptadecanoic acid as a standard and was extracted with 0.5 ml of chloroform-methanol (2:1, v:v). The organic phase was recovered and fatty acid methyl esters were produced using HCl (1 M) in methanol. Samples were analyzed by capillary gas chromatography-mass spectrometry and quantitation of fatty acid methyl esters was done by normalizing peak areas (of total ion current) to the heptadecanoate standard.

2.7. Immunoblot analysis

Protein samples were resolved by conventional SDS-gel electrophoresis [20] and transferred to nitrocellulose for immunoblot analysis using alkaline phosphatase or ECL detection as described before [3–5]. Densitometry of immunoblots used an Epson scanner and the MacBas program from Fuji.

3. Results

3.1. Alkaline sodium carbonate and CAPS stripping of membranes

It was previously reported that pH 11.5 sodium carbonate stripped a significant proportion of *Drosophila* csps from membranes [16]. However, in *Torpedo*, pH 11.5 CAPS solution did not strip csps from membranes [13]. To examine

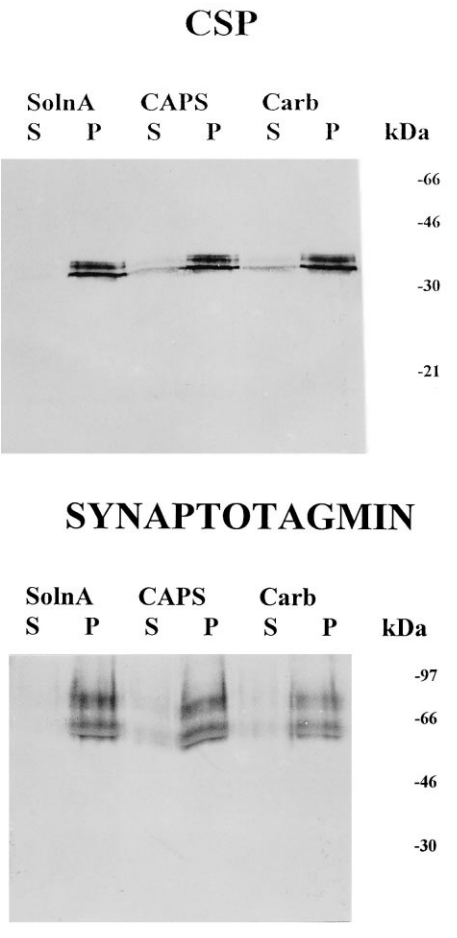

Fig. 1. Alkaline treatment of *Drosophila* head membranes. Membranes were suspended in solution A (soln A) or in 0.1 M solutions of sodium carbonate (Carb) or CAPS at pH 11.5. After 1 h at 4°C, membranes were sedimented, and csp and synaptotagmin immunoreactivity were determined in the supernatant (S) and pellet (P). Note that synaptotagmin immunoreactivity is split between bands of about 55 kDa and 70 kDa, as reported previously [10]. The mobility of molecular mass markers is indicated here, and in subsequent figures (mass in kDa).

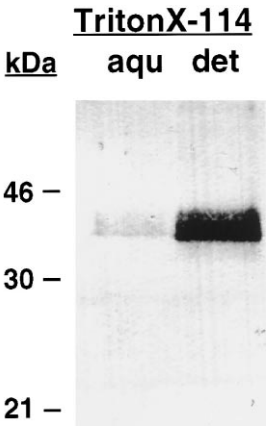

Fig. 2. Triton X-114 partitioning of *Drosophila* csps. Triton X-114 partitioning was performed as described in Section 2 and csp immunoreactivity was determined for the aqueous supernatant (aqu) and the detergent bead (det).

further this situation, we assessed the effects of solution A, CAPS and carbonate on the membrane association of *Drosophila* csps. As a control, we also monitored the distribution of synaptotagmin immunoreactivity, because synaptotagmin is a well-established integral membrane protein of vertebrate and invertebrate synaptic vesicles [18,22,23]. As shown in Fig. 1, when *Drosophila* membranes were resuspended in solution A, no detectable csp or synaptotagmin immunoreactivity was displaced from the membranes into the supernatant. In other words, during a second round of sedimentation, all of the detectable csps (and, synaptotagmin) was pelleted (Fig. 1). However, alkaline CAPS and carbonate both stripped csps from *Drosophila* head membranes (Fig. 1). These results (Fig. 1) were typical of the proportion of csp immunoreactive protein that was seen in the supernatant after exposure of membranes to these alkaline solutions. However, what was more important for this study was that synaptotagmin immunoreactivity was also partially solubilized by these same alkaline solutions (Fig. 1). In all of our experiments, the proportion of synaptotagmin that was displaced from membranes by CAPS or carbonate essentially paralleled the amount of displaced csps (Fig. 1). Densitometric analysis of the quantity of csps displaced from membranes by CAPS or carbonate yielded values of $10 \pm 3\%$ and $12 \pm 4\%$, respectively (mean ± S.E. for $n = 4$). For synaptotagmin the comparable results were $8 \pm 4\%$ and $10 \pm 4\%$, respectively. Because of these results, and since synaptotagmin is generally regarded as an integral membrane protein of synaptic vesicles [18,22,23], we conclude that these alkaline solutions were partially solubilizing intrinsic membrane proteins of *Drosophila* secretory vesicles. Moreover, the similar effects of CAPS and carbonate suggest that this effect is due more to alkaline pH rather than a specific action of carbonate.

To explore further the issue of whether alkaline carbonate solution was partially solubilizing *Drosophila* membranes, we evaluated the extent to which carbonate displaced long-chain fatty acids from these membranes relative to solution A, which is at pH 7.5. In two separate experiments (with duplicate samples, so $n = 4$) we detected the release into solution A of 200–700 ng of each of the following long-chain fatty acids: palmitate, palmitoleate, stearate, oleate and linoleate. 4–10 times as much of each of these fatty acids was detected in supernatant samples of carbonate-treated membranes. From these results, we conclude that the mild, solubilizing effect of carbonate on integral membrane proteins seen in Fig. 1, also culminates in the release of long-chain fatty acids from these *Drosophila* head membranes.

3.2. Triton X-114 partitioning of Drosophila csps

Triton X-114 partitioning was developed by Bordier [21] as an empirical strategy to assess the hydrophobic character of proteins. As shown in Fig. 2, when Triton X-114 partitioning was performed on csps solubilized from fruit fly heads, almost all of the immunoreactive csps associated with the detergent bead. It is important to note for these experiments that the partitioning behavior of *Drosophila* csps was strongly affected by the protein:detergent ratio. Thus, results similar to those of Fig. 2 were obtained when we solubilized two or fewer fly heads per 0.1 ml of 1% Triton X-114. Increasing amounts of csp immunoreactivity appeared in the supernatant when we

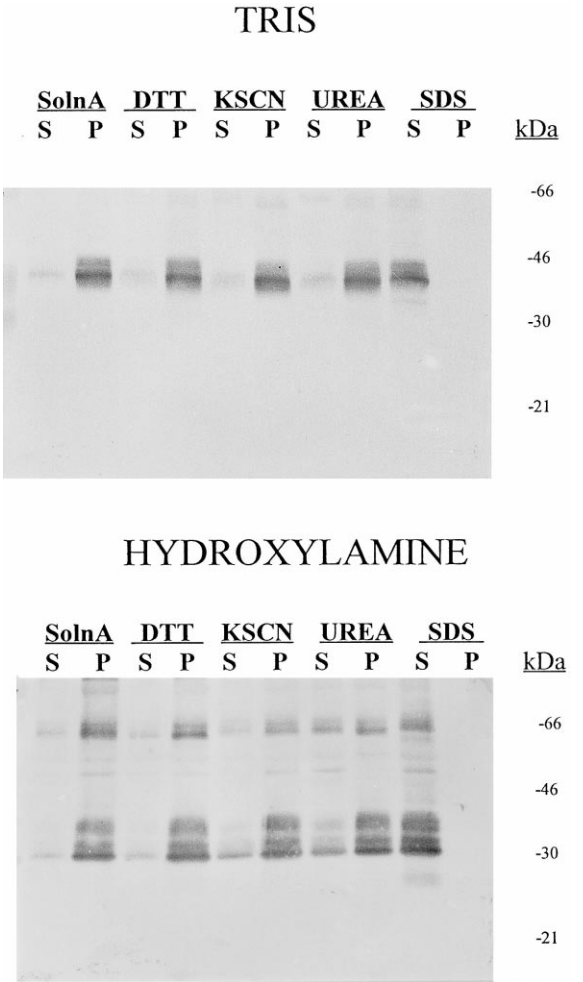

Fig. 3. Chemical treatments of *Drosophila* head membranes. *Drosophila* membranes were suspended in solution A supplemented with 1 M Tris (pH 7.0) or 1 M hydroxylamine (pH 7.0–7.2). Other additions to this solution included: (i) 50 mM DTT (DTT lanes); (ii) 2.5 M KSCN and 50 mM DTT (KSCN lanes); (iii) 8 M urea and 50 mM DTT (urea lanes); or (iv) 0.2% SDS in 50 mM DTT (SDS lanes). After overnight incubation, membranes were sedimented and csp immunoreactivity was assayed in supernatants (S) and pellets (P) of control samples and those with the additions indicated above.

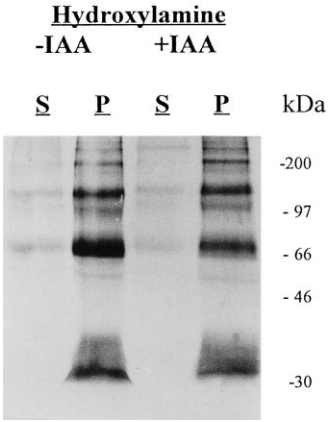

Fig. 4. Efforts to displace deacylated csps from membranes using iodoacetic acid. *Drosophila* head membranes were incubated in 1 M hydroxylamine in solution A (final pH 7.2) without (−) or with (+) iodoacetic acid (50 mM). After 16 h at 22°C membranes were sedimented and csp immunoreactivity was assayed in the supernatant (S) and pellet (P).

used three or more fly heads in this volume (data not shown). We conclude that the results of Fig. 2 are consistent with the hypothesis that csps are integral membrane proteins that partition efficiently into Triton X-114 condensates.

3.3. Chemical treatments of Drosophila membranes

Van de Goor and Kelly [16] made the unexpected observation that *Drosophila* csps remained membrane associated even after treatment with 1 M hydroxylamine (pH 7) had removed thioester-linked fatty acyl residues (deacylation was confirmed by the shift in electrophoretic mobility of the csp immunoreactive protein). Because hydroxylamine treatment is, by necessity, conducted under conditions of high salt (in our experience, more than 0.8 mole equivalents of NaOH must be added per each mole equivalent of hydroxylamine hydrochloride to achieve neutral pH), we investigated the type of interaction(s) that might contribute to the persistent membrane association of deacylated csps. Our treatments of *Drosophila* membranes were designed to assess whether deacylated csps could be displaced from membranes under conditions that disrupt general classes of interactions between biological macromolecules. For these experiments, control membranes were incubated in 1 M Tris (pH 7.0) in place of 1 M hydroxylamine.

The first treatment that we used was 50 mM DTT. As an effective reagent for reducing disulfide bonds of proteins [24], DTT should release any csp that was linked to other membrane components via such bonds. As shown in Fig. 3, DTT did not significantly displace acylated or deacylated csps from membranes. Moreover, there was no perceptible change in the distribution of acylated or deacylated csps relative to membranes incubated without DTT (Fig. 3). From these data, we conclude that even though deacylation of *Drosophila* csps should expose all 15 of the thiol moieties of the cysteine string of these proteins, these thiols do not *covalently* attach csps to membranes under these circumstances.

Because hydroxylamine treatment involves conditions of high salt, we regarded it as unlikely that csp was electrostatically associating with membranes. However, to test further this possibility, membranes were suspended in hydroxylamine solution plus 2.5 M KSCN (Fig. 3). While this treatment displaced a small quantity of deacylated csps (compared to acylated csps in Tris, Fig. 3) into the supernatant, this effect was not particularly dramatic relative to samples in solution A that contained no KSCN (Fig. 3). Thus, neither acylated nor deacylated csps were efficiently stripped from membranes under these conditions. From this, we conclude that the forces binding deacylated csps to these membranes are largely insensitive to alkali metal cations or chaotropic anions.

To assess the role of hydrogen bonds in the membrane association of csps, we used 8 M urea (Fig. 3). Like KSCN, urea caused slightly more csp immunoreactive protein to appear in the supernatant fraction relative to membranes in solution A alone (Fig. 3). However, the vast majority of csp immunoreactive protein still sedimented after urea treatment (Fig. 3). From these results, we conclude that it is not hydrogen bonding that underlies the persistent membrane association of deacylated csps.

As a final treatment, we used 0.2% SDS, which efficiently

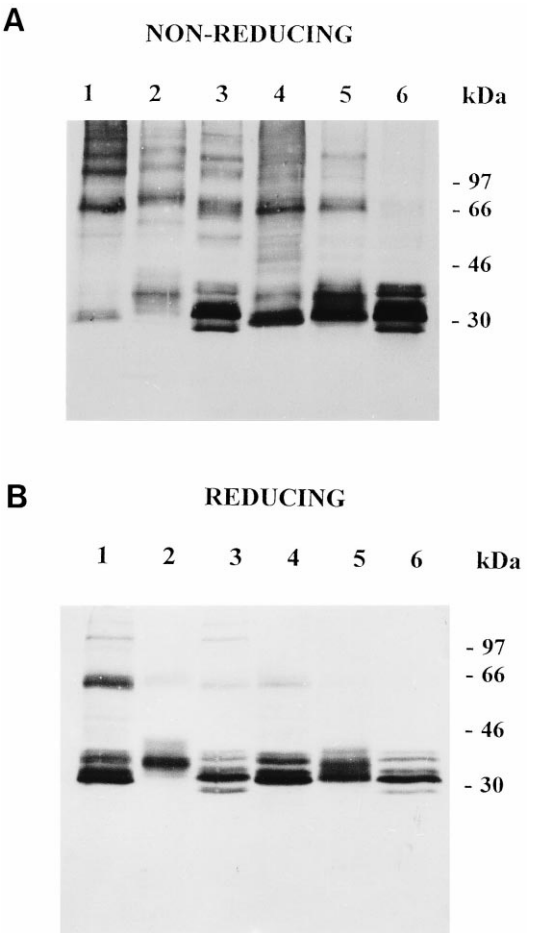

Fig. 5. Accessibility of csp thiol groups to alkylation by iodoacetic acid or iodoacetamide in membrane or detergent environments. *Drosophila* head membranes were incubated in the absence (lanes 1–3) or presence (lanes 4–6) of SDS (1%) in 1 M hydroxylamine with further additions to the solution as indicated: lanes 1 and 4, no addition; lanes 2 and 5, iodoacetic acid; lanes 3 and 6, iodoacetamide. After overnight incubation at 22°C, samples either received no DTT (non-reducing) or DTT (50 mM, reducing) and were extracted for immunoblot analysis. For samples in B, DTT (50 mM) was added to the sample buffer for electrophoresis.

solubilized both acylated and deacylated csps (Fig. 3). In separate experiments, 10 mM CHAPS worked equally well to solubilize csps (data not shown). These results indicate that both acylated and deacylated csps associate with membranes and that detergent disruption of the membranes is necessary to solubilize these proteins. Subsequent experiments examined the basis of this interaction of deacylated csps with membranes.

3.4. Studies of the membrane association of deacylated csps

Because detergent treatment was the only strategy that effectively solubilized deacylated csps from membranes (Fig. 3), we inferred that these csps interacted hydrophobically with some component of the membrane. It thus became reasonable to ask which domain of csps mediated this hydrophobic interaction. Prior hydrophobicity analyses [1] had revealed that the only significantly hydrophobic domain of csps was its cysteine string region. Thus, we hypothesized that this domain might contribute to the persistent membrane association of deacylated csps. To test this hypothesis, we conducted two additional sets of experiments. First, we reasoned that if iodoacetic acid were capable of alkylating thiol residues of csps during the process of hydroxylamine-mediated deacylation, the accumulation of negative charge in this otherwise hydrophobic region (due to acetic acid covalently bound to thiol residues) might displace csps from membranes. This experiment was a modification of the procedures used in Fig. 3. The results we obtained are in Fig. 4. When *Drosophila* head membranes were incubated with hydroxylamine in solution A in the absence or presence of iodoacetic acid, we saw no significant change in the extent of displacement of csp immunoreactivity from the membranes into the supernatant (Fig. 4). However, at the same time, the membrane-associated csps showed extensive multimerization, regardless of whether or not iodoacetic acid was present (Fig. 4). This multimerization was due predominantly to thiol cross-linking of deacylated csps (which was largely reversed if DTT was included in the sample buffer, data not shown for these experiments, but see Fig. 5). On the basis of these results, we provisionally concluded that the thiol groups of csps were inaccessible to iodoacetic acid, and therefore were not alkylated under the conditions of the experiment in Fig. 4. One explanation for this failure of iodoacetic acid to alkylate the newly exposed thiol groups of these csps is that these thiols are embedded in a hydrophobic milieu that is not efficiently penetrated by iodoacetic acid. If this interpretation is correct, it favors the idea that the cysteine string of csps is buried in a hydrophobic environment subsequent to deacylation.

Our provisional interpretation of the failure of iodoacetic acid to alkylate csps in the experiment in Fig. 4, was that the thiol groups of csps were inaccessible to this reagent. If this were the case, we postulated that the neutral alkylating reagent, iodoacetamide, should be capable of alkylating csps and inhibiting the multimerization of these proteins revealed in Fig. 4. Thus, for this set of experiments, we treated *Drosophila* head membranes with hydroxylamine and either iodoacetic acid or iodoacetamide. In the absence of alkylating reagent, we saw the typical multimerization of csps (Fig. 5A, lane 1). When iodoacetic acid was present during the deacylation (as in Fig. 4), it somewhat attenuated the overall immunoreactive signal, and slightly shifted the mobility of the immunoreactive csp (Fig. 5A, lane 2). We suspect that both the mobility shift and diminished immunoreactivity may reflect the ability of iodoacetic acid to target amino groups of proteins as well as thiols [25]. However, the important point here is that, as in Fig. 4, iodoacetic acid did not efficiently preclude multimer formation by csps that remain in the membrane environment (Fig. 5A, lane 2). However, iodoacetamide was much more effective in this context. Thus, as indicated (Fig. 5A, lane 3), when deacylation of membrane-associated csps occurred in the presence of iodoacetamide, this reagent suppressed multimer formation and left appreciably more of the csp in the monomeric form (this is seen in the immunoreactive cluster at about 30 kDa in Fig. 5A, lane 3). This differential susceptibility of csp to alkylation by iodoacetamide versus iodoacetic acid is consistent with the idea that the thiol groups of csps are in a hydrophobic milieu (that can be penetrated by iodoacetamide, but not by iodoacetic acid). The results from conducting this same experiment in the presence of SDS (to solubilize csps), supports this conclusion (Fig. 5A, lanes 4–6). Thus, when deacylation was conducted in SDS, we detected a reduction of csp multimer formation even in the absence of alkylating agents (Fig. 5A, lane 4). We infer from this that when csps are dispersed in micelles, the incidence of multimer formation is decreased. Moreover, inclusion of iodoacetic acid or iodoacetamide further suppressed multimer formation (Fig. 5A, lanes 5 and 6). This was most noticeable with iodoacetamide which almost completely eliminated any higher mass immunoreactive species (Fig. 5, lane 6). Again, these data are compatible with the idea that the thiol groups of the cysteine string of csps prefer an apolar environment (the interior of a biological membrane or a detergent micelle) subsequent to deacylation.

Finally, to verify that the putative thiol-linked multimers of csp seen in Figs. 4 and 5A do indeed derive predominantly from thiol cross-linking, we repeated the experiments of Fig. 5A, but treated the samples with DTT prior to the organic-solvent extraction protocol, and we included DTT in the sample buffer. As indicated in Fig. 5B, this strategy largely eliminated the csp multimerization phenomenon. Interestingly, a substantial amount of the deacylated, dimeric csps persisted in membranes incubated in the absence of alkylating reagent (Fig. 5B, lane 1). However, in all other conditions (Fig. 5B, lanes 2–6) there was either very little or no detectable dimeric csp. These results indicate that the high density of thiol groups in csps (11 in a row in *Drosophila*, see [1]) makes these proteins particularly prone to multimerize and that this susceptibility is enhanced when these thiol groups are in a hydrophobic environment. More importantly, these results (Fig. 5B) show that the complex pattern of immunoreactive csp multimers in Fig. 5A can be reduced to an interpretable pattern by DTT.

4. Discussion

These investigations were undertaken to clarify the nature of the membrane association of csps in *Drosophila*. Our observations indicate that csps in *Drosophila*, as in *Torpedo* [13], are integral membrane proteins. Moreover, we investigated the basis of the persistent membrane association of csps that had been deacylated by hydroxylamine. Our results are consistent with the hypothesis that the deacylated cysteine string domain of csps mediates this persistent membrane association. These results are important because they constrain the func-

tional contributions of csps to membrane surfaces where these proteins appear to play one or more important roles in regulated secretory events [4].

It was previously suggested [16] that *Drosophila* csps were peripheral membrane proteins, because alkaline sodium carbonate stripped a portion of csps from *Drosophila* membranes. In our studies, alkaline carbonate and CAPS solutions both displaced *Drosophila* csps from membranes. However, in neither solution was more than about 15% of the csp immunoreactivity dislodged from these membranes. Moreover, we also found that there was a parallel displacement from these membranes of fatty acids, and the synaptic vesicle protein, synaptotagmin. Because synaptotagmin is acknowledged to be an integral membrane protein [21–23], these data suggest that alkaline solutions have a mild solubilizing activity on these fruit fly membranes. From these results, we provisionally conclude that *Drosophila* csps are integral membrane proteins that are partially solubilized by alkaline solutions.

Results from Triton X-114 partitioning experiments provided further support for the conclusion that csps are integral membrane proteins in *Drosophila*. The original work of Bordier [21] established that integral membrane proteins partition preferentially into the detergent phase after thermal condensation of aqueous solutions of Triton X-114. In our studies, *Drosophila* csps efficiently extracted into the detergent phase in Triton X-114 partitioning experiments. These observations were not entirely unexpected given that both we [26] and van de Goor and Kelly [16] had concluded that *Drosophila* csps, like *Torpedo* csps [14], were extensively fatty acylated. This high degree of covalent lipidation of csps (estimated to be 11 or 12 moles of lipid per mole of csps in *Torpedo* [14]) requires that if these proteins are to be solubilized from membranes, there must be some mechanism to shield the fatty acyl moieties from the aqueous milieu. The principal pathway that is available for this shielding to occur in Triton X-114 partitioning experiments is for csps to extract into the detergent phase. The agreement between this prediction and our empirical results strengthens the conclusion that csps are integral membrane proteins.

We confirmed the previous observation [16] that *Drosophila* csps remain membrane associated even after chemical deacylation by hydroxylamine. However, our interpretation of these results is strongly influenced by the subsequent experiments we performed. First, it should be noted that hydroxylamine-mediated deacylation of membrane-associated csps was achieved under highly non-physiological conditions. In our hands, more than 0.8 mole equivalents of NaOH was needed to neutralize hydroxylamine hydrochloride. Thus, the 1 M solution of hydroxylamine that we (and others [16]) employed also had about 0.8 M NaCl. Relative to *Drosophila* hemolymph or common physiological salines used for *Drosophila* [27], this hydroxylamine solution is excessive both in its salt concentration and osmotic pressure. In spite of this, we found that we could not dislodge deacylated *Drosophila* csps from hydroxylamine-treated membranes unless we disrupted the membranes using detergent. Reducing agent, urea, and the chaotropic anion, thiocyanate, all failed to displace these csps. These results indicate that the persistent membrane association of deacylated csps does not rely on disulfide bonds, hydrogen bonds or electrostatic interactions. Instead, we inferred that the persistent membrane association of deacylated csps was due to a hydrophobic interaction between csps and some component of the membrane. This led us to explore which domain(s) of csps mediated this hydrophobic interaction. We addressed this issue in a final series of experiments.

In general, csps are very hydrophilic proteins [4], and the only region of sustained hydrophobicity includes the cysteine string [1,2]. For this reason, we focused on the cysteine string as the candidate domain most likely to mediate the persistent membrane association of deacylated csps. Evidence supporting such a role for the cysteine string comes from our experiments showing that deacylated *Drosophila* csps resist alkylation by iodoacetic acid, but not by iodoacetamide. These results are consistent with the idea that the cysteine residues remain in a hydrophobic environment that is accessible to iodoacetamide, but not iodoacetic acid (which is charged at neutral pH). While additional work will be necessary to establish unequivocally that the cysteine string mediates this interaction, it is our assertion that this work is of secondary importance to studies of the membrane association of csps under physiologically more relevant conditions. Thus, both this group [14] and van de Goor and Kelly [16] found that recombinant, unacylated csps remained soluble in living cells and that it was only the fatty acylated csps that became membrane associated [14]. These latter results lend credence to our contention that it is the non-physiological conditions of hydroxylamine treatment that promote the membrane association of deacylated csps. It will be of considerable interest to establish whether fatty acylation of csps is both necessary and sufficient for their anchoring to membranes in vivo.

In summary, our investigations have shown that *Drosophila* csps, like *Torpedo* csps [13], are integral membrane proteins. This conclusion is important, because it means that there is no species difference in the nature of the membrane association of csps. Moreover, these results imply that the main functional role(s) of csps in secretion must be constrained by the intrinsic membrane association of these proteins. Thus, both the putative role of csps as modulators of presynaptic calcium channels [28], and the hypothetical involvement of csps in membrane fusion [29] must take into account this membrane anchoring of csps.

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