

Structure–function analysis of the cysteine string protein in *Drosophila*: cysteine string, linker and C terminus

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Summary

Cysteine string proteins (CSPs) are conserved secretory vesicle proteins involved in regulating neurotransmitter and peptide release. While the function of the J-domain has been studied in detail, little is known about other conserved regions. We have constructed mutant genes coding for proteins with modified cysteine string, linker region or C terminus and transformed them into *Csp* null-mutant *Drosophila*. In the living animal, mutated CSP lacking all cysteines fails to associate with membranes, does not concentrate in synaptic terminals, and cannot rescue adult temperature-sensitive paralysis and short life span, both prominent null mutant phenotypes. A mutant

protein with 5 instead of 11 string cysteines appears to be normally targeted but cannot rescue paralysis at 37°C. We propose that the cysteine string, in addition to its role in targeting, may be essential for a function of CSP that is dependent on the number of cysteines in the string. A deletion in the linker region or the C terminus does not affect CSP targeting, and function in adults is only marginally impaired.

Key words: cysteine string protein (CSP), secretory vesicle, *Drosophila*, *in vitro* mutagenesis, protein targeting, paralysis, life span.

Introduction

Cysteine string proteins (CSPs) are unique cysteine-rich proteins present on synaptic vesicles and secretory organelles (Buchner and Gundersen, 1997; Chamberlain and Burgoyne, 2000; Zinsmaier and Bronk, 2001). The characteristic and conserved regions in the primary structure of invertebrate and vertebrate CSPs comprise an N-terminal phosphorylation site for protein kinase A (PKA), a J-domain, a cysteine string (CS) consisting of 13–15 cysteines within a stretch of 25 amino acids, a ‘linker’ region connecting J and CS, and a less conserved C-terminal domain.

Phosphorylation of vertebrate CSP at Ser¹⁰ reduces its interaction with syntaxin and may thus modulate exocytosis (Evans et al., 2001). The J-domain has been linked to a putative co-chaperone function of CSPs. CSPs bind to and stimulate the ATPase activity of the molecular chaperone HSC70 only if the J-domain is intact (Braun et al., 1996; Chamberlain and Burgoyne, 1997). In *Drosophila* the *Csp* and *hsc70-4* genes interact (Bronk et al., 2001). Recently, the small glutamine-rich tetratricopeptide repeat protein (SGT) has been identified as a third component of this chaperone complex (Tobaben et al., 2001). The function of the cysteine string has been controversial. The extensive palmitoylation of the cysteine string suggested a role in membrane attachment

(Gundersen et al., 1994); however, depalmitoylation does not displace CSPs from membranes (van de Goor and Kelly, 1996; Chamberlain and Burgoyne, 1998), indicating that the hydrophobic amino acids associated with the cysteine string are sufficient to keep the protein attached to the membrane (Mastrogiovanni et al., 1998). Replacement of seven of the 14 cysteines in PC12 and HeLa cells demonstrated that the intact string is required for initial membrane targeting in cultured cells (Chamberlain and Burgoyne, 1998). Information on the function of the linker region and the C terminus is sparse so far. The inhibitory effect on regulated exocytosis of overexpressing the mammalian CSP2 isoform in hamster insulinoma cells is reduced by a point mutation in the linker region. The C-terminal difference between the two known vertebrate isoforms appears to be critical for this suppression (Zhang et al., 1999). Linker and/or cysteine string appear to be important for CSP-CSP self-association (Swayne et al., 2003).

Deletion of the *Csp* gene from the *Drosophila* genome causes semi-lethality during prolonged development, a severely shortened life span of escapers, temperature-sensitive paralysis, and breakdown of evoked synaptic transmission at elevated temperatures (Umbach et al., 1994; Zinsmaier et

Materials and methods

Wild-type flies and transgenic mutants were raised on standard food medium at 18°C and kept over balancer TM6,Tb,Sb unless otherwise noted. The *Csp^{UI}* mutant was generated by unequal crossing over between two P(⁺) elements flanking the *Csp* gene and thus contains a P(⁺) element in place of this gene. In the *Csp^{UIw}* mutant this P(⁺) element has been removed by remobilization (Eberle et al., 1998). Both strains are in *w¹¹¹⁸* background. To eliminate genetic modifiers from the *Csp^{UI}* strain these (red-eyed) flies were out-crossed to *w¹¹¹⁸* for 12 generations and selected for the red eye color marker of the P(⁺) element to generate the strain *Csp^{UIoc}*, which was then maintained over TM6,Sb,Tb balancer.

Antibodies were obtained from the following sources. Anti-*Drosophila* CSP: DCSP1 is mouse monoclonal antibody (mAb) ab49 (Hofbauer, 1991; Buchner et al., 1986), DCSP2 (6D6) and DCSP3 (1G12) were kindly provided by K. Zinsmaier. Anti-*Drosophila* SAP47: mouse mAb nc46 (Hofbauer, 1991; Reichmuth et al., 1995). Anti-*Drosophila* syntaxin: 3D8 was kindly provided by S. Benzer (Caltech, Pasadena, CA, USA) and later obtained from Developmental Studies Hybridoma Bank (DSHB), University of Iowa, USA.

The QuickChange™ site-directed mutagenesis kit was obtained from Stratagene (Cambridge, UK). Restriction enzymes were purchased from Life Technologies (Karlsruhe, Germany) and Fermentas MBI (St Leon-Rot, Germany). Enhanced chemiluminescence antibody binding detection kit was obtained from Amersham Corp. (Slough, UK). Oligonucleotides were synthesized by MWG (Ebersberg, Germany).

Csp cDNA-1 ['lcz49-9'; Zinsmaier et al. (1990); 'type-I'; Zinsmaier et al. (1994)], was cleaved by *Bsp*1407I and *Eco*RI and the resulting 1.9 kb fragment containing 3' sequences of exon 3 and exons 4-7. This fragment was ligated to a *Hind*III/*Bsp*1407I (long) or an *Asp*718/*Bsp*1407I (short)

For site-directed mutagenesis the type-I cDNA was used as template in the QuickChange™ system. The deletion of 11 cysteines was achieved in two steps. The forward primers (reverse = complement) used were 5'-CACTGGATGCTG-TTGTTCGCTGCAACTTCTGCTGC-3' for the first step resulting in the deletion of six cysteines in the construct *Scsp* (short cysteine string protein), and 5'-TGTGCCGTGATCAC-TGGAAACTTCTGCTGCGG-3' for the second step, leading to the construct *Cslp* (cysteine string less protein). The conversion of the two flanking pairs of cysteines to serines was again obtained in two steps. The primers for this mutation were 5'-AAGGCGGTGGTTATCTCCTCTGCCGTGATCA-CTGGA-3' and its complement for the first step and 5'-TGA-TCACTGGAAACTTCTCCTCCGGCAAGTTCAAGC-3' and its complement for the second step, to obtain the construct *Clp* (cysteine less protein). For the replacement of the cysteine string by a serine string in the *Ssp* (serine string protein) construct the primers were 5'-CGTGATCACTGGATCCTC-TTCTTCCTCCTCCTCCTCCTCCTCTTCCAACCTTCTGCT-GCGG-3' and its complement. Deletion of the eight amino acids AEQFGEEN in the linker region of the *LΔ8* construct was achieved by the primer 5'-CGGCTTGACATAGTC-AACGCATACTTCGTGGTCACTTCACCG-3' and its complement. In order to eliminate 27 C-terminal amino acids in the *CA27* construct the codons for the amino acids PVAA were replaced by TAGTAGTAGCT, introducing three in-frame stop codons and a frame shift.

To confirm the fidelity of the introduced mutations the DNA of all mutant clones was sequenced prior to transformation. For transformation into the genome of *Drosophila* the modified cDNA was cleaved and ligated to the putative genomic promoter/enhancer region of *Csp* gene as described above for the rescue vector *lcDna1*.

Heads of wild-type and mutant *Drosophila* were either directly homogenized in 1 μl head⁻¹ of sample buffer (Laemmli, 1970) or collected on ice and homogenized with 5 μl head⁻¹ buffer A (150 mmol l⁻¹ NaCl, 10 mmol l⁻¹ Hepes, pH 7.4, 1 mmol l⁻¹ EGTA, 0.1 mmol l⁻¹ MgCl₂). A post-nuclear supernatant was obtained by 1000 *g* centrifugation for 10 min at 4°C. The supernatant was subjected to a 100 000 *g* centrifugation for 60 min at 4°C to separate soluble proteins from proteins attached to membranes or cytoskeleton. For deacylation pellets were resuspended in 1 mol l⁻¹ hydroxylamine (pH 7) and incubated for 20 h at room temperature. After incubation, an additional 60 min centrifugation step at 100 000 *g* and 4°C followed in order to

separate proteins solubilized by the treatment (S3) from the membrane fraction (P3). Pellets were finally dissolved in equivalent volumes of sample buffer, and proteins from equal volumes of supernatants and dissolved pellets were separated on 12.5% gels by SDS-PAGE followed by western blot analysis using monoclonal antibodies. Separation of different membrane fractions was achieved by glycerol gradient velocity sedimentation as described by van de Goor et al. (1995). All experiments were done at least twice.

Immunohistochemistry

Immunohistochemistry was carried out as described earlier (Buchner et al., 1986; Ashburner, 1989). The mouse monoclonal supernatants were applied at dilutions of 1:10 to 1:100. For visualizing antibody binding the ABC detection system (Vector Labs, Burlingame, USA) was used. Western blots were incubated in monoclonal supernatants at 1:100 dilution and developed using the ECL detection system as recommended by the manufacturer.

Epitope mapping

The epitopes of the three anti-CSP monoclonal antibodies used in this study (DCSP1-3) were mapped by spotting sequential decapeptides on nitrocellulose membranes according to a procedure described in detail elsewhere (Munch et al., 1999), followed by spot detection using ECL as in western blots. Amino acids common to decapeptides binding the antibody are defining the epitope.

Behavioral analysis

Temperature sensitivity of the various genotypes was scored by transferring groups of 10–15 1-day-old flies in empty food vials to a water bath of defined temperature and counting fully paralyzed animals after 5 min. To prevent interference of adaptive phenomena all flies were tested only once. Effects of variations in genetic background were minimized by using flies that had recently been crossed into the out-crossed null mutant *Csp^{Uloc}*.

Survival analysis

Normalized life span was determined by transferring groups of 10 F₁ siblings from heterozygote crosses to fresh food vials at 25°C and counting dead flies daily.

Electrophysiology

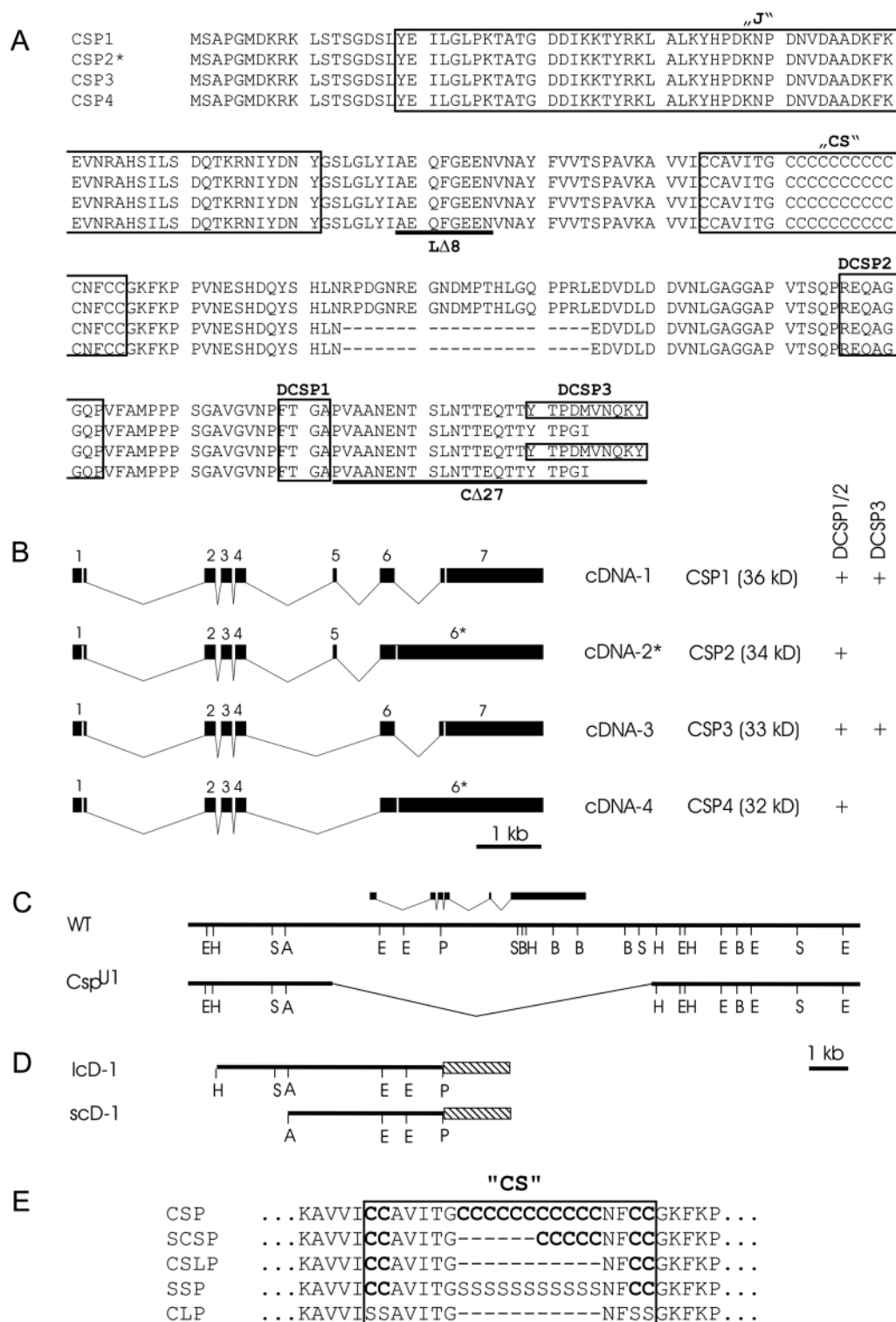
For electrophysiological recordings climbing unbalanced third instar larvae were immobilized for 20 min on ice. Preparation and recording essentially followed the procedure described by Stewart et al. (1994). Larvae were dissected in an Elastasil® coated Petri dish under modified HL3 Ringer containing 1 mmol l⁻¹ Ca²⁺ starting with mid-dorsal incision. 'Filets' were pinned out flat and internal organs were removed carefully. After transfer to a temperature-controlled fixed microscope stage, intracellular recordings were made from ventral longitudinal muscle 6 of abdominal half-segment 3 using quartz glass microelectrodes of 20–30 MΩ resistance,

prepared by a laser-based microelectrode puller (P-2000, Sutter Instrument Co., Novato, CA, USA) and filled with 3 mol l⁻¹ KCl. Excitatory junctional potentials (EJPs) were evoked by stimulating the distal portion of the motor nerve taken up into a fire-polished borosilicate suction electrode. Stimulus amplitude of 0.1 ms pulses was adjusted to a value 2.5-fold of the initial threshold. Signals were amplified with Neuroprobe Amplifier (Model 1600, A-M Systems, Inc., Carlsborg, WA, USA), low-pass filtered at 50 kHz, and digitized via an A/D interface (Data Acquisition Processor DAP 3200e/315, Microstar Laboratories, Inc., Bellevue, WA, USA). Stimuli, signals and temperature were digitized at 10 kHz, data were analyzed with DASyLab Ver. 5.03.30 (measX, GmbH and Co. KG, Mönchenglabbach, Germany). Temperature was measured by a temperature sensor (Newport Electronics GmbH, Deckenpfronn, Germany), positioned in the Ringer solution near the larval filet. For each preparation six evoked EJPs were averaged 5–7 min after reaching the set temperature. If more than six stimuli produced no response the amplitude was registered as 'zero'. Each experiment was performed with at least four larvae. Statistical analysis was performed using GraphPad Instat™ Ver. 2.04a (GraphPad Software, San Diego, CA, USA) and Statistica Ver. 6 (StatSoft, Inc., Tulsa, OK, USA).

Results

In *Drosophila* four different CSP isoforms (denoted as CSP1–CSP4 in Fig. 1) are observed in western blots of head homogenates (Zinsmaier et al., 1994), while only the largest isoform (CSP1) is detected in non-neural organs (Eberle et al., 1998). No information is presently available on the functional differentiation of the various isoforms or the relevance of their selective distribution in the brain (Eberle et al., 1998; Nie et al., 1999). Three different cDNAs have been isolated so far (Zinsmaier et al., 1990, 1994). In order to assign these cDNAs to the isoforms detected in western blots we mapped the epitopes of the three available monoclonal antibodies against *Drosophila* CSPs, mAbs DCSP1, DCSP2 and DCSP3, by oligopeptide spotting. The boxes marked 'DCSP1', 'DCSP2', and 'DCSP3' in Fig. 1A comprise those amino acids that are common to all decapeptide spots recognized by the respective antibody. The data demonstrate that mAbs DCSP1 and DCSP2 recognize all cDNA-encoded proteins while mAb DCSP3 binds only to the isoforms encoded by cDNA-1 and cDNA-3 (Zinsmaier et al., 1994; Eberle et al., 1998). We speculate that a hitherto undetected fourth splice variant (cDNA-2*) may code for the second largest isoform recognized in western blots, as indicated in Fig. 1B, which summarizes cDNA structures, approximate isoform sizes, and antibody recognition in western blots.

The main aim of the present work was to determine the involvement of the cysteine string, the linker region, and the C terminus of *Drosophila* CSP in membrane association, synaptic vesicle targeting and function. We first tested whether a single isoform (CSP1) expressed in *Csp* null mutants can



'rescue' the mutant phenotype. To this end we designed two different wild-type *Csp* cDNA 'rescue' gene constructs, *scDna1* and *lcDna1*. The various transgenic mutants generated by transforming *in vitro* mutagenized versions of these cDNA constructs into *Csp*-null background can then be compared to the 'rescue' transformants in order to assess the effect of the mutations on CSP function.

cDNA-1 rescues all major phenotypes of *Csp* null mutants

We used cDNA-1, which contains the largest open reading frame. Since the regulatory region of the *Csp* gene has not yet been characterized we tested two genomic fragments of different lengths upstream of the gene as depicted in Fig. 1D for their capacity to direct wild-type expression of the cDNA. Because there was evidence that the first intron may contain

Fig. 1. (A) Primary structure of the three cDNA-derived cysteine string protein isoforms CSP1, CSP3 and CSP4, and the postulated isoform CSP2*. The conserved 'J' domain, the cysteine string region ('CS'), and the epitopes recognized by the three available monoclonal antibodies DCSP1, DCSP2 and DCSP3 are boxed, the amino acids deleted in the mutant proteins LΔ8 and CΔ27 are underlined. (B) Schematic representation of the exon-intron structure of the three known *Csp* transcripts cDNA-1, cDNA-3 and cDNA-4, and the hypothetical cDNA-2*. The white vertical lines in exons 1, 6* and 7 indicate start and stop codons, which delimit the open reading frames. The approximate molecular masses in SDS gels and the recognition (+) of the four isoforms by the monoclonal antibodies DCSP1, DCSP2 and DCSP3 are indicated. (C) Genomic region of the *Csp* locus and the extent of the deficiency of the *Csp^{U1w}* null mutant compared with wild type (WT). (D) cDNA-1 derived rescue constructs *lcDnal* (*lcD-1*) and *scDnal* (*scD-1*). The constructs consist of genomic fragments of different sizes (compare with C) containing regulatory sequences and exons 1, 2 and part of exon 3, as well as introns 1 and 2, and a common cDNA-1 fragment (hatched) containing the rest of exon 3, and exons 4, 5, 6 and 7. The depicted constructs and their *in vitro* modified versions (cf. A and E) were cloned into the pW8 P-element vector. Restriction enzymes: A=Asp718, B=BamH I, E=EcoRI, H=HindIII, P=Bsp1407I, S=SalI. (E) Amino acid sequence of the cysteine string region of *Drosophila* CSP and the cysteine string mutant proteins 'short cysteine string protein' (SCSP), 'cysteine string-less protein' (CSLP), 'serine string protein' (SSP) and 'cysteine-less protein' (CLP). Cysteine residues (C) are shown in bold.

important *Csp* regulatory sequences (K. E. Zinsmaier, personal communication), fragments were chosen that extend from 2.3 kb (*scDnal*) or 4.5 kb (*lcDnal*) upstream of the transcription start to a *Bsp1407I* site in the third exon and thus include the first two introns. These genomic fragments were ligated to the cDNA-1 fragment downstream of the *Bsp1407I* site (hatched in Fig. 1D). The constructs were transformed into the germ line of *Drosophila* wild type. Since no difference has been observed in the expression or function of the two constructs, both are referred to below as construct *cDnal*. In western blots of head homogenates from *cDnal* transgenic flies the monoclonal antibody DCSP-1 detects a highly overexpressed largest CSP isoform in addition to the three other wild-type isoforms (data not shown). Surprisingly, we do not observe any of the phenotypes described for flies overexpressing CSP by use of the UAS/GAL4 system (cf. Discussion). Head homogenates of flies homozygous for the *cDnal* transgene and the null allele *Csp^{U1w}* produce only a single very strong signal on western blots. This signal matches in size the largest isoform of wild-type homogenates, and in fractionation and deacylation experiments behaves like all four isoforms of wild-type flies (Figs 2, 3, and data not shown). Immunohistochemistry employing mAb DCSP1 on head and body sections of these flies shows an expression pattern of CSP1 both in nervous and non-nervous tissues that is indistinguishable from wild-type stainings (Eberle et al., 1998), with high concentrations of CSP1 in synaptic terminals on muscles and in the neuropil of brain and thoracic ganglia (Fig. 4 and data not shown). All tested *cDnal* transformants

rescue the premature death (Fig. 5A) and the temperature-sensitive paralytic phenotype (Fig. 6) of the null mutant *Csp^{U1}*, demonstrating that the 2.3-kb upstream genomic region and the first two introns contain all essential regulatory regions of the *Csp* gene and that the CSP1 isoform is sufficient to maintain all known functions of the four natural isoforms. Thus *cDnal* constructs can be used as targets for introducing site-directed mutations into the *Csp* gene *in vitro* in order to study the effects of such mutations *in vivo* after transformation of the modified gene into *Csp^{U1}* flies. *cDnal* transformants and *Csp^{U1}* null mutants serve as positive and negative controls, respectively.

Generation of transgenic flies with deletions in the linker, cysteine string and C-terminal regions of CSP

Mutation of conserved amino acids in a protein is likely to modify its function. We therefore designed *Csp* transgene constructs coding for the following modified proteins: a variant with a deletion of eight amino acids (underlined in Fig. 1A) in the linker region (LΔ8), four different variants with mutated cysteine string (Fig. 1E), and a variant with a deletion of the 27 C-terminal amino acids (underlined in Fig. 1A; CΔ27). In the 'short cysteine string protein' (SCSP) variant, six amino acids of the cysteine string are deleted while the entire string of 11 cysteines is removed in the 'cysteine string-less protein' (CSLP) variant. In the variant 'serine string protein' (SSP), these 11 cysteines are replaced by 11 serines. Serine is a small, uncharged amino acid with structural similarity to cysteine. Replacement of the cysteine string by a serine string possibly produces smaller overall structural changes in the protein than its deletion. In the variant 'cysteine-less protein' (CLP) the 11 string cysteines have been deleted and the two flanking pairs of cysteines mutated to serines (Fig. 1E) such that the encoded protein contains no cysteines. Transgenic flies with these constructs were generated as described above for the *lcDnal* construct and were crossed into *Csp^{U1w}* and *Csp^{U1oc}* mutant background (cf. Materials and methods for difference between these two mutant strains).

Analysis of the mutated proteins

Head homogenates of wild-type and transgenic flies in *Csp^{U1w}* null mutant or wild-type background were analyzed on immunoblots stained with DCSP1 and DCSP2 antibody (Figs 2, 3). No qualitative differences were observed between blots stained with these two antibodies except for the CΔ27 isoform, which is not recognized by DCSP1. Apparently, the epitope at the very C-terminal end of the truncated protein cannot bind the antibody (Fig. 1A). The calculated molecular masses of the serine string protein (SSP), the short cysteine string protein (SCSP), the cysteine string-less protein (CSLP) and the cysteine-less protein (CLP) differ from the largest wild-type isoform CSP1 only by 0.176, 0.726, 1.331 and 1.395 kDa, respectively. The apparent molecular mass as seen in the SDS gel, however, is approximately 6, 4, 7 and 7 kDa smaller, respectively, compared to the 36 kDa CSP1 isoform. This discrepancy is presumably due to the fact that most

cysteines of wild-type CSP are palmitoylated. Indeed, the untreated SSP migrates at about the same position as the deacylated 36 kDa CSP1 isoform or the largest deacylated isoform of wild-type head homogenate (Fig. 2C). Hydroxylamine treatment of S2 supernatants of SSP and CLP did not alter apparent molecular masses demonstrating that

these proteins are not significantly acylated (data not shown). The C-terminal deletion of 27 amino acids leads to a shift in western blots of about 5 kDa, somewhat larger than the calculated loss of 3.868 kDa. In contrast, the protein with a deletion in the linker region (LΔ8) shows the expected approximate molecular mass difference of 1.048 to the 36 kDa wild-type isoform (Fig. 2A). Note that the strength of the western signals of all mutated proteins except CΔ27 is strongly reduced (Fig. 2A; cf. Discussion).

In order to test if the mutated proteins associate with membranes, homogenates of heads from transformants and wild type were separated into cytosolic and membrane fractions and analyzed by immunoblotting (Fig. 2B). In contrast to the wild-type CSP isoforms from wild-type flies or *cDna1* transformants, the CLP and SSP mutant forms can be detected only in the soluble fraction. This is confirmed by

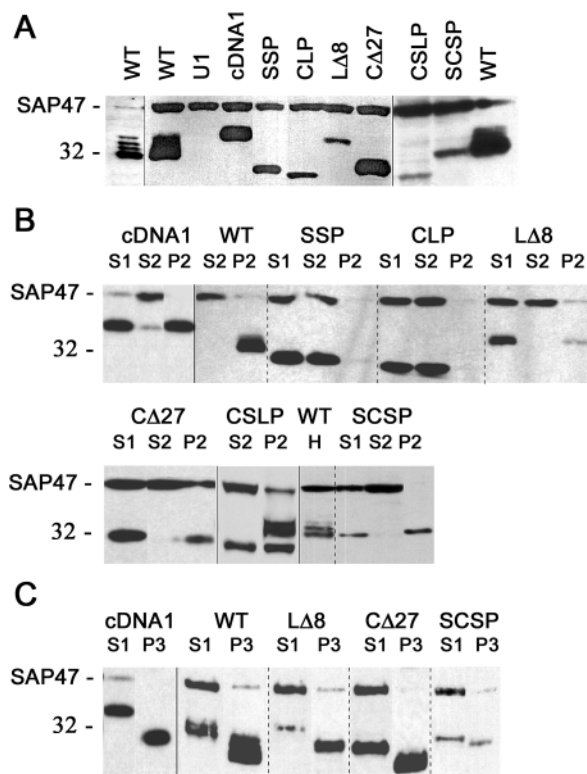


Fig. 2. Immunoblots of (white-eyed) wild type (WT), null mutant *Csp^{U1w}* (U1), *cDna1*-rescued null mutant (cDNA1), and transgenic mutants (serine string protein (SSP), cysteine-less protein (CLP), linker deletion (CΔ8), C-terminal deletion (CΔ27), cysteine string-less protein (CSLP), and short cysteine string protein (SCSP), all except for CSLP in B in *Csp^{U1w}* genetic background. Vertical dotted lines separate lanes of different mutants and vertical solid lines separate different blots. The SAP47-marked signals represent loading controls. Blots were developed with mAbs DCSP1 (DCSP2 when CΔ27 was loaded) and mAb nc46 for detection of SAP47. (A) Heads homogenized in SDS buffer, 1–2 heads per lane. The leftmost WT lane was from a large gel for improved separation and has been graphically compressed for comparison with the small gel lanes. (B) Heads were homogenized in buffer A, a post-nuclear supernatant (S1) was fractionated by ultracentrifugation to separate soluble proteins (S2) from membrane or cytoskeleton associated proteins (P2). Wild-type CSPs (WT, cDNA1), LΔ8, CΔ27 and SCSP are detected exclusively in the membrane fraction, CSLP (analyzed here in WT background as control) is present in both fractions, whereas CLP and SSP are seen only in the soluble fraction. A single WT head homogenized in SDS buffer is shown for comparison (H). (C) Pellets P1 were deacylated with hydroxylamine and ultracentrifuged to obtain supernatant S3 and pellet P3. A smaller protein after deacylation (P3) indicates the loss of palmityl residues in wild-type CSPs (WT, cDNA1), LΔ8, CΔ27 and SCSP. 32, position of the 32 kDa marker protein.

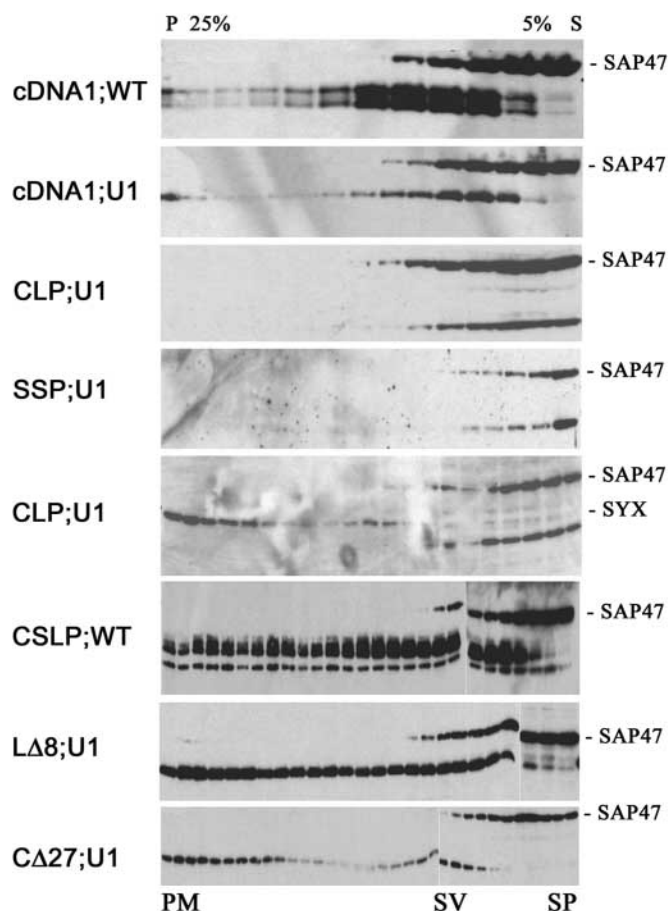


Fig. 3. Glycerol gradient velocity sedimentation of wild-type CSPs (cDNA1), CLP, SSP, CSLP, LΔ8 and CΔ27 in null-mutant (U1) and wild-type (WT) backgrounds. S, supernatant; P, pellet, SP soluble proteins; SV, synaptic vesicles; PM, plasma membrane. The gradient was allowed to develop overnight from 5% to 25% in 5% steps. Wild-type CSPs migrate in the synaptic vesicle fractions whereas SSP and CLP comigrate with the soluble control protein SAP47. A significant portion of CSLP, LΔ8 and CΔ27 appears to comigrate with the plasma membrane fraction identified by the PM protein syntaxin (SYX).

glycerol gradient velocity sedimentation experiments using *cDna1*, *Ssp* and *Clp* flies in *Csp^{U1w}* and wild-type background (Fig. 3). Endogenous or transgenic wild-type CSPs migrate predominantly in the synaptic vesicle fractions (van de Goor et al., 1995) whereas SSP and CLP comigrate with the loading control protein SAP47, which is soluble (Reichmuth et al., 1995). Transgenic cysteine string-less protein CSLP, on the other hand, is found in similar concentrations in soluble and membrane fractions (Fig. 2B). The protein with the short cysteine string (SCSP) is found in the pellet, similar to the wild-type isoforms. The fractionation of wild-type CSPs is apparently not significantly influenced by the overexpression in the same cells of the CSP1 isoform in a wild-type background. The proteins with intact cysteine string but deletions in the linker or C-terminal domains, LΔ8 and CΔ27, are found in the synaptic vesicle fractions and, compared to wild-type CSPs, appear to be somewhat enriched in the plasma membrane fractions (Figs 2, 3). Fractions containing plasma membranes are identified in the gradient by use of an antibody against syntaxin (mAb 8C3, SYX in Fig. 3).

Tissue distribution of mutated proteins

In wild-type flies, CSPs are highly concentrated in synaptic neuropil, synaptic terminals on muscles, and various non-nervous structures (Eberle et al., 1998). No differences in the immunohistochemical staining patterns have been observed between wild-type flies and *cDna1*;*Csp^{U1w}* transformants (Fig. 4A,B, and data not shown). In these experiments head sections of wild-type and transgenic flies were stained with mAb DCSP1 on the same microscope slide to ensure identical immunohistochemical treatment. Comparison of Fig. 4C with D and F reveals the selective disruption of synaptic terminal targeting of the cysteine string mutant proteins SSP and CLP in the respective transformants in null mutant background. In *Ssp*;*Csp^{U1w}* or *Clp*;*Csp^{U1w}* the modified proteins distribute rather homogeneously throughout all parts of the brain, including cellular rind, axonal bundles (e.g. in the optic chiasms) and neuropil (Fig. 4D,F). In the *Cslp*;*Csp^{U1w}* transformant a certain fraction of the modified protein is apparently targeted to the synaptic terminals while the remaining fraction distributes to other parts of the neurons (Fig. 4E). The deletion of six cysteines of the string (SCSP) (Fig. 4G).

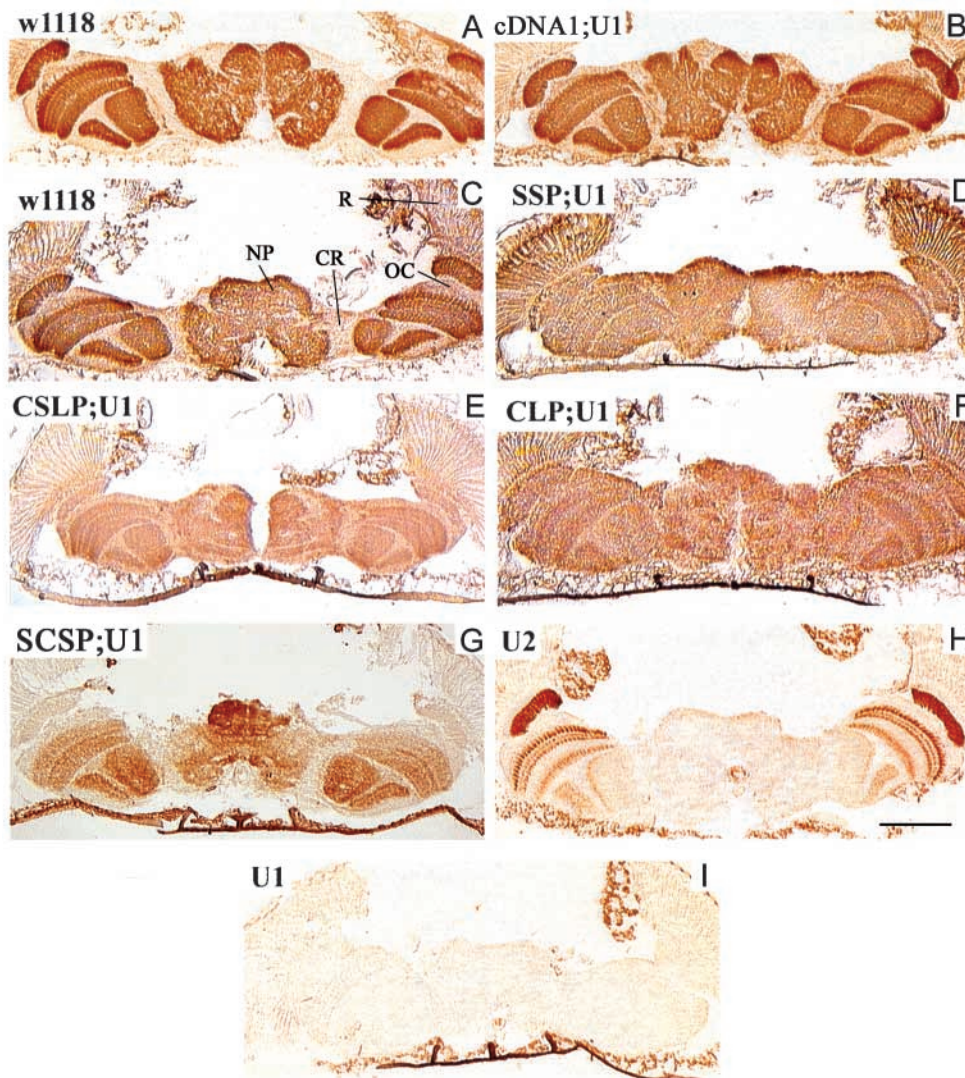


Fig. 4. Distribution of wild-type or mutated CSP forms in wild-type (w1118; A,C), and in *cDna1*–, *Ssp*–, *Cslp*–, *Clp*– and *Scsp*–transformed flies in *Csp^{U1}* null mutant background (B,D–G), and in hypomorphic mutant *Csp^{U2}* (H). Horizontal frozen head sections. Sections A+B, C+D, were stained on the same microscope slides and processed identically for better comparison. There are no clear differences in staining between wild type (A) and *cDna1*;*Csp^{U1}* rescue (B). SSP (D) and CLP (F) distribute homogeneously throughout cellular rind (CR) or retina (R), fiber tracts such as the optic chiasms (OC) and neuropil (NP). CSLP (E) apparently is targeted to synaptic neuropil less efficiently than WT CSPs. *Scsp*;*Csp^{U1}* preparations (G) demonstrate low abundance of SCSP but normal targeting of this isoform to synaptic neuropil. Specificity of staining is demonstrated by comparison with the null mutant *Csp^{U1}* (I). Scale bar, 100 μ m.

(Fig. 4G) or of eight amino acids in the linker region (LΔ8) (not shown) strongly reduces staining intensity but does not alter the distribution of the protein. Deletion of the C terminus (CΔ27) (not shown) does not affect immunohistochemical staining patterns. For comparison we show stainings of two mutants. The *Csp*^{U2} mutation (Eberle et al., 1998) leaves the coding region intact but carries a B104 transposon insertion in intron 4 of the *Csp* gene. This mutant shows selective CSP accumulation in mushroom body neuropil (not shown) and in synaptic terminals of photoreceptors and visual interneurons (Fig. 4H), but CSP expression in the rest of the brain is drastically reduced. The *Csp*^{U1} null mutant serves as a negative control (Fig. 4I), demonstrating that fat body staining is unspecific.

Phenotypes of cysteine-string mutant transformants

Longevity

A severely reduced life span of adult flies has been observed previously as a major phenotype of *Csp* null mutants. We noted that quantitative aspects of this and other phenotypic traits of *Csp*^{U1} flies, such as the degree of developmental delay and semi-lethality, the critical temperature for the failure of larval synaptic transmission or adult paralysis, all significantly

depend on genetic background. The originally semilethal *Csp*^{U1} and *Csp*^{U1w} stocks (Eberle et al., 1998) that had been kept at 18°C in the homozygous condition could after several years be maintained at 25°C, larvae no longer showed reversible synaptic failure in nerve muscle preparations at 32°C, and adults lived significantly longer than observed previously. We therefore out-crossed for 12 generations the *Csp*^{U1} stock against *white* (*w*¹¹¹⁸; *Csp*⁺ in Canton-S background) until we noted that homozygous flies again showed the strong phenotype described earlier (Umbach et al., 1994; Zinsmaier et al., 1994). To further reduce the influence of genetic background we performed the experiments analyzing phenotypes of the transformants on siblings whenever possible. With P(tg) transgene insertions on the X chromosome (tg=*cDna1*, *Ssp*, *LΔ8* or *CΔ27*), crosses of TM3 balanced flies heterozygous for the out-crossed *Csp*^{U1oc} mutation and the P insertion of the transformation (all in *w*⁻ background) were used:

$$\frac{w, P(tg)}{w} \ddot{::} \frac{Csp^{U1oc}}{TM3} \times \frac{w, P(tg)}{Y} \ddot{::} \frac{Csp^{U1oc}}{TM3}.$$

where TM3 denotes the balancer chromosome and Y the Y-chromosome.

The survival functions at 25°C for the balanced flies (one copy of the *Csp*⁺ gene), the homozygous *Csp*^{U1oc} flies and the four P transgenics in homozygous *Csp*^{U1oc} background are shown in Fig. 5A. Obviously, the dramatic life-shortening phenotype of the *Csp*^{U1oc} mutation is rescued by the wild-type *cDna1* construct and by the constructs with a deletion in the linker region and in the C terminus, *LΔ8* and *CΔ27*. Flies with only the *Ssp* construct, on the other hand, live merely 1 day longer than the null mutants. Life expectancy (50% survival time) of *white* flies at 25°C is about 35 days under these conditions. For characterization of the *Clp* transformant, which mapped to the third chromosome and was recombined with the original *Csp*^{U1w} mutation, siblings from the following crosses were analyzed:

$$\frac{w}{w} \ddot{::} \frac{P(Cl p), Csp^{U1w}}{P(Cl p), Csp^{U1w}} \times \frac{w}{Y} \ddot{::} \frac{Csp^{U1oc}}{TM6},$$

$$\frac{w}{w} \ddot{::} \frac{Csp^{U1w}}{Csp^{U1w}} \times \frac{w}{Y} \ddot{::} \frac{Csp^{U1oc}}{TM6}.$$

In the F₁ generation we can compare longevity of *Csp*⁺ (balanced) flies and *Csp*-null flies of mixed genetic background (*Csp*^{U1w}/*Csp*^{U1oc}), with or without the *Clp* transgene. The survival curves for these four genotypes are shown in Fig. 5B. Again, the construct without cysteines (*Clp*) cannot rescue the short life phenotype and increases life expectancy of the null mutant only by 10–20%. Transformants with *Cslp* and *Scsp* constructs in *Csp*^{U1} background could not be tested for longevity because several independent transformant lines were difficult to maintain, indicating that these constructs may produce a dominant negative effect (cf. Discussion). Life expectancy of the hypomorphic *Csp*^{U2} mutant at 18°C is about 44 days, compared to 12 days for *Csp*^{U1} and 88 days for wild

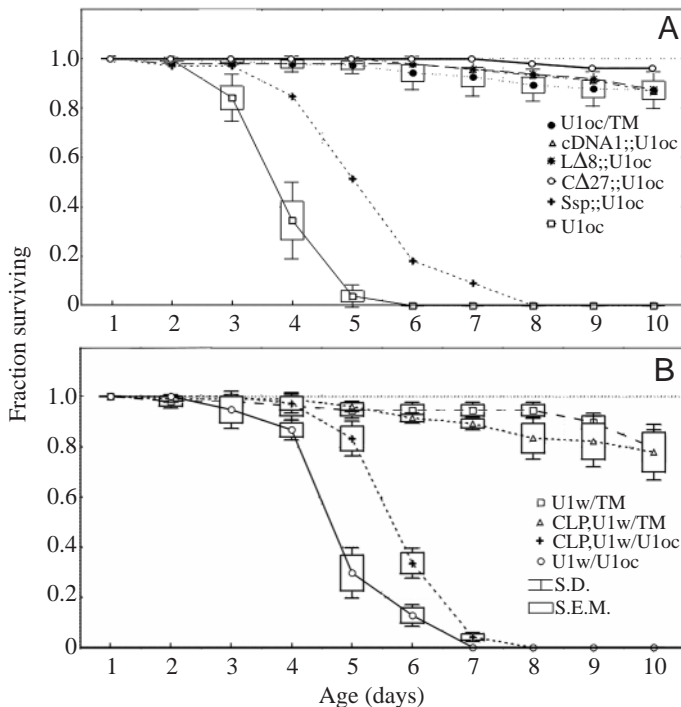


Fig. 5. (A) Survival rates of homozygous *Csp*^{U1oc} mutants (U1oc/U1oc), balanced siblings (U1oc/TM) and *cDna1*-, *LΔ8*-, *CΔ27* or *Ssp*-transformed flies in null mutant background at 25°C culture temperature. In contrast to the wild-type *Csp*, *LΔ8* or *CΔ27*, transgenic expression of *Ssp* cannot rescue the short-life phenotype of null mutants and extends their life time only by ca. 20%. (B) Survival time for *Clp*- and *Ssp*- transformed flies are similar. Note the slightly increased survival time of *Csp*^{U1}/*Csp*^{U1oc} compared to *Csp*^{U1oc}/*Csp*^{U1oc} in A.

type under identical conditions. This demonstrates that small amounts of intact CSP can sustain much of its vital function whereas relatively normal expression of CLP or SSP is almost ineffective for sustaining the life of adults.

Temperature-sensitive paralysis of adult flies

Paralysis at elevated temperatures represents a second prominent phenotypic aspect of *Csp* null mutants. Temperature sensitivity was quantified as shown in Fig. 6 for wild type ($w^{1118};Csp^+$), two null mutant lines (*Csp*^{U1w} and *Csp*^{U1oc}), and five transgenic lines in *Csp*^{U1oc} background (*cDna1*, *LΔ8*, *CA27*, *Clp*, *Ssp*). For the transgenic lines, each curve represents the average of two independent insertion lines that did not differ significantly. The difference between the two null mutants reflects the influence of genetic background mentioned above (cf. Discussion). This phenomenon makes it rather difficult to interpret the small difference between the two null mutants. Yet it is clear that the *CA27* and *LΔ8* constructs essentially rescue temperature-sensitive paralysis whereas constructs with mutations in the cysteine string do not. A weak dominant negative effect is apparent in the two independent insertions of the *Clp* construct in the *Clp*;*Csp*^{U1oc} lines. Qualitative tests of the few surviving adult *Cslp*;*Csp*^{U1w} and *Scsp*;*Csp*^{U1w} transformants reveal temperature-sensitive paralysis similar to the null mutant. Again the hypomorphic *Csp*^{U2} mutant displays a much weaker phenotype than the string mutants as it paralyzes at 37°C only after 6–10 min.

Electrophysiology

The influence of the various mutations in the *Csp* transgene on neuromuscular transmission has been studied by intracellular recordings from body wall muscle 6 of third instar

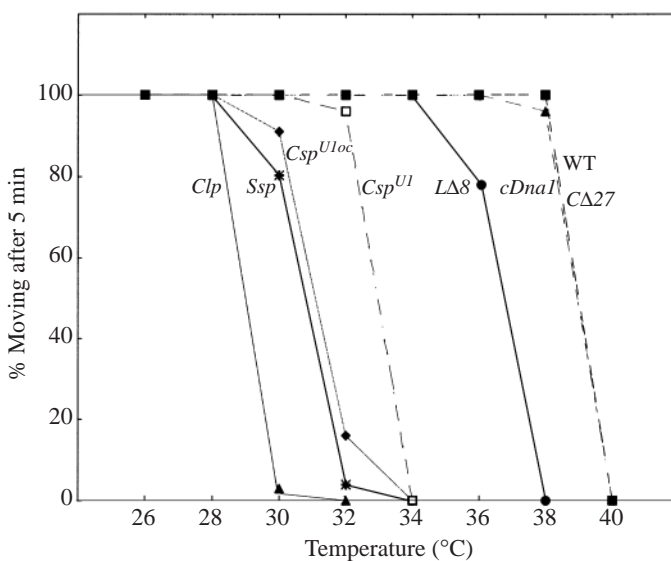


Fig. 6. Temperature-sensitive paralysis of adult wild type (WT), null mutant (*Csp*^{U1}), and *cDna1*–, *LΔ8*–, *CA27*–, *Ssp*–, or *Clp*–transformed flies in null mutant background. Note again the difference between *Csp*^{U1} and *Csp*^{U1oc}.

larvae. The motor neurons innervating this muscle were cut and stimulated by a suction electrode. All transgenes were analyzed in homozygous *Csp*^{U1oc} genetic background. The results in Fig. 7 demonstrate that replacement of the cysteine string in *Ssp*;*Csp*^{U1oc} transgenic mutants causes significantly reduced excitatory junction potential (EJP) amplitude at 18°C

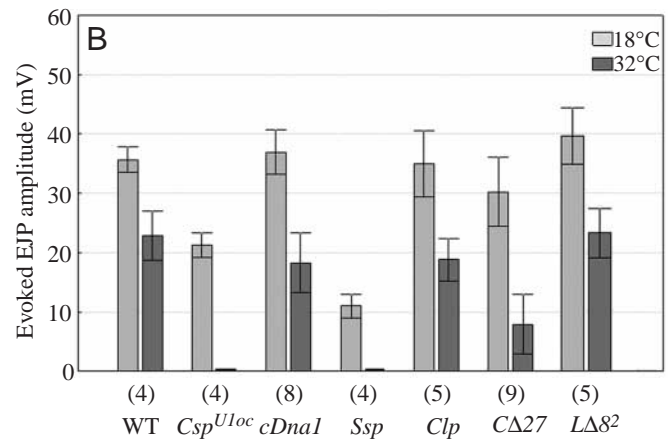
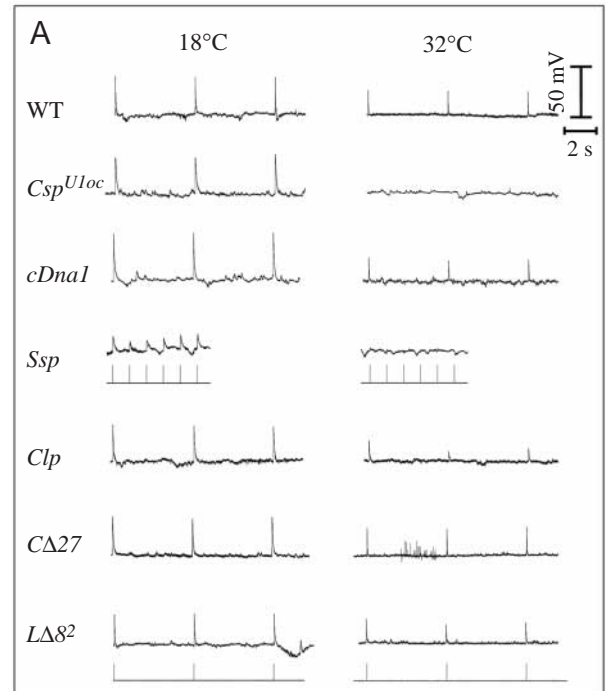


Fig. 7. (A) Evoked excitatory junction potentials (EJPs) recorded from larval nerve-muscle preparations of WT, null mutant *Csp*^{U1oc}, and *cDna1*–, *Ssp*–, *Clp*–, *LΔ8*–, *CA27*–transformed animals in *Csp*^{U1oc} null mutant background. Data were pooled for at least two independent transgene insertions except for *LΔ8*¹ and *LΔ8*², which differed significantly (see Results). The two independent *CA27* transformants display normal transgene expression, indicating that this construct does not fully rescue the larval temperature-sensitive phenotype, whereas adults of these lines show no obvious defects. (B) Evoked EJP amplitudes (mean ± S.E.M.) of (*N*) preparations.

and break-down of synaptic transmission at 32°C, similar to the null phenotype (Umbach et al., 1994). (Control experiments with wild type and null mutant had shown that responses were similar at 0.2 and 1.0 Hz). Contrary to expectations on the basis of the biochemical data and adult phenotypes, larval neuromuscular transmission in *Clp;Csp^{U1oc}* larvae persisted in four out of five preparations while the fifth preparation showed the expected null mutant phenotype. Behavioral observation verified the paralysis of intact *Ssp;Csp^{U1oc}* transgenic larvae at 32°C while *Clp;Csp^{U1oc}* larvae appeared almost unaffected by this temperature. Again unexpectedly, synaptic transmission failed at 32°C in three out of seven transgenic *CΔ27;Csp^{U1oc}* larvae. Equivocal results were obtained with two independent insertion lines of the *LΔ8* construct. *LΔ8¹* responses were similar to those of the null mutant, *LΔ8²* showed wild-type-like EJPs. It was noted that expression of the transgene was strongly reduced in *LΔ8¹* larvae compared to *LΔ8²* larvae but similar in adults of the two lines. It is therefore assumed that synaptic failure in *LΔ8¹* larvae is due to low expression levels rather than the deletion in the linker region.

Discussion

The Csp locus

Sequence annotation of the *Drosophila* genome project has identified two genes in close proximity upstream (CG11523) and downstream (CG11489) of the *Csp* gene. Possible influences of these genes on the data presented here have to be considered. Based on the gene prediction data, the *Csp^{U1}* but not the *Csp^{X1}* deletion (Eberle et al., 1998) could affect the downstream gene. However, northern blots using EST clones of both genes as probes (Reisch, 2003) did not reveal any differences between *w¹¹¹⁸*, *Csp^{U1}* or *Csp^{X1}*, and no phenotypic differences between *Csp^{U1}* and *Csp^{X1}* have so far been described (disregarding some weak immunohistochemical staining in the subesophageal ganglion in *Csp^{X1}*; Eberle et al., 1998). We conclude that if transcripts of the adjacent genes are affected by the *Csp^{U1}* mutation they must be of low abundance and have no obvious influence on adult nervous system function.

Linker region and C terminus are not involved in membrane binding

CSPs from the electric organ of *Torpedo* were shown to be membrane associated, and the demonstration that at least 11 residues of the cysteine string were thioester-linked with palmitic acids suggested that this highly hydrophobic region was responsible for the observed properties of CSPs as integral membrane proteins (Gundersen et al., 1994; Mastrogiacomo et al., 1994). Injection of unmodified CSPs into frog oocytes supported this notion, but deacylation of membrane proteins in experiments using PC12 cells, HeLa cells or *Drosophila* heads failed to release CSPs into the supernatant (van de Goor and Kelly 1996; Chamberlain and Burgoyne 1998). These findings suggested that the palmitoylated cysteine string is not required

for stable membrane attachment of CSPs and it was proposed that a different domain might be responsible for membrane association (Chamberlain and Burgoyne 1998; van de Goor and Kelly 1996). None of the mutations introduced in the present work outside the cysteine string resulted in a release of membrane-bound CSP variants into the supernatant during a deacylation experiment. Thus neither the linker nor the C-terminal domain are relevant for membrane attachment of deacylated CSP.

A cDNA transgene construct coding for the largest CSP isoform rescues the null mutant

In the present study two wild-type *Csp* cDNA gene constructs were designed and transformed into *Csp* null-mutant flies. Both constructs express the 36 kDa CSP1 isoform. In the wild type this isoform represents the sole CSP in non-neural tissues, whereas in the brain three additional smaller isoforms of 34, 33 and 32 kDa are expressed more abundantly (Eberle et al., 1998). On sections of *cDna1*-rescued null mutants the distribution of CSP as detected by mAb DCSP1 in neuronal (and non-neuronal, data not shown) tissues matches the stainings described for wild-type flies. We conclude that the essential sequences for regulation of CSP expression are contained in a region from 2.3 kb upstream of the transcription start to the end of the second intron of the *Csp* gene. Both *cDna1* constructs rescue the salient features of the null-mutant phenotype, i.e. temperature-sensitive failure of synaptic transmission and early death. Thus expression of the 36 kDa isoform is sufficient to rescue the major cellular functions of the *Csp* gene. The cell-specific expression of alternatively spliced CSP isoforms in the brain (Zinsmaier et al., 1994; Eberle et al., 1998) may fine-tune synaptic transmission to achieve yet unknown functional specializations. The strong overexpression of the 36 kDa isoform in head homogenates of *cDna1* transformants is likely to result predominantly from the absence of alternatively spliced introns such that all transcripts translate into the same protein isoform. This overexpression of the 36 kDa isoform has been analyzed in 12 independent strains with different insertion sites and does not cause any obvious new phenotypes, in either null-mutant background or when co-expressed with the three smaller isoforms in the wild type (Fig. 2B and data not shown). Nie et al. (1999) demonstrate rescue of the attenuated excitatory junction potential (EJP) amplitude of *Csp^{U1}* larval nerve muscle preparations by expressing any of the three known cDNAs under control of the *elav* promoter (mediated by the GAL4-UAS system), but find dramatic effects on eye and wing development and on survival rate by overexpressing these cDNAs. Since CSP overexpression from the *Csp* promoter in genomic rescue transformants (Zinsmaier et al., 1994; Nie et al., 1999) or our *cDna1* transformants does not produce these effects, we propose that they may be caused by higher levels of overexpression or temporal and/or spatial misexpression due to the *elav* GAL4 driver.

Phenotypic analysis of mutants or transgenic animals has to take into consideration the influence of genetic background

effects. The loss of both semi-lethality and larval synaptic failure at 32°C in the *Csp^{U1}* null mutant cultured in the homozygous condition, indicates that genetic modifiers suppressing *Csp*-related phenotypes have been selected for. Extensive out-crossing against wild type was required to re-establish a line that showed phenotypic characteristics similar to the original null mutant. This suggests that important genetic modifiers may be present on the third chromosome in the vicinity of the *Csp* gene. All electrophysiological experiments and most behavioral tests were therefore performed on flies in the out-crossed *Csp^{U1oc}* null mutant background.

Mutated CSPs cannot fully restore normal function

To elucidate the role of the linker region, the cysteine string (CS), and the C-terminal domain in CSP function, we analyzed expression and membrane association of six mutant isoforms in transgenic flies as well as the associated phenotypes. For each construct several independent transformants were obtained, and at least two were tested in each experiment in order to detect possible effects of the site of transgene insertion. Deletion of the entire cysteine string (11 out of 15 cysteines of the CS region) results in a protein (CSLP) that is found both in the soluble and membrane fractions. Replacement of the 11 cysteines by serines in SSP, however, eliminates membrane targeting. These results suggest that the relative positions of the remaining two pairs of cysteines may be critical for the residual targeting of CSLP. The SSP and the CLP are detected only in the soluble fraction and are not enriched in synaptic terminals. An earlier study used a mammalian mutant CSP construct in which 7 of the 14 cysteines of the string region were replaced by serines. When this mutant protein was expressed in PC12 or HeLa cells, it was not palmitoylated and was not associated with membranes (Chamberlain and Burgoyne, 1998). Our data make clear that the cysteine string is required for correct targeting of *Drosophila* CSPs to synaptic vesicles in an intact organism. In *Drosophila* the short cysteine string mutant protein (SCSP), in which 6 out of 15 cysteines in the string region have been deleted, appears to be efficiently targeted to membranes. A small increase in electrophoretic mobility is observed after depalmitoylation of SCSP. In spite of partial (CSLP) or normal (SCSP) targeting to membranes, transgenic flies expressing these proteins in null mutant background display paralysis similar to the null mutant, strongly indicating that an intact cysteine string region is necessary for cellular functions of CSPs beyond protein targeting. Such functions may, for example, depend on the tightness of membrane attachment by the palmitoylated cysteine residues. The fact that several transgenic lines containing the *Cslp* or *Scsp* constructs were difficult or impossible to maintain in homozygous *Csp^{U1w}* genetic background indicates that mutant CSPs with intact or partially intact targeting but modified or deleted cysteine string disturb cellular processes that can be maintained in the absence of CSPs. SSP and CLP, on the other hand, are not targeted but slightly improve viability.

The present experiments demonstrate that for the most visible consequences of CSP function in adult *Drosophila* an intact linker region and the C terminus are not essential. Both regions contain amino acids that are highly conserved in evolution, demonstrating fitness-related function. The temperature sensitivity of neuromuscular transmission in some *CA27;Csp^{U1oc}* larvae could relate to such a function and indicates that in larval synapses CSPs may function differently compared to adult synapses. The unexpected 'rescue' of synaptic transmission and prevention of paralysis at 32°C by CLP in most larvae but not in adults also points in this direction. The intra-strain variability of some of the electrophysiological data might be due to polymorphic genetic background effects, which can never be entirely eliminated. The only defect noticed in the *LA8²;Csp^{U1oc}* line concerns a moderate temperature sensitivity of adults. The synaptic failure in *LA8¹;Csp^{U1oc}* larvae is assumed to be caused by the low larval expression of the transgene in this strain due to its specific insertion site.

Western blot signals from head homogenates of all mutated proteins except CA27 are significantly weaker. Since this effect is independent of transgene insertion sites it is assumed that it reflects reduced stability of the mutated proteins. Instability of mutated or truncated proteins is a general phenomenon. The normal stability of the CA27 protein is not unexpected because a natural mammalian CSP isoform lacks the conserved C-terminal domain (31 amino acids; Chamberlain and Burgoyne, 1996; Coppola and Gundersen, 1996). We asked whether the reduced abundance of the mutated proteins might simply be responsible for the phenotype of the transgenic flies in *Csp*-null background. However, the hypomorphic mutation *Csp^{U2}*, which reduces CSP abundance about tenfold but leaves the coding region intact, displays strongly attenuated phenotypes compared to null mutants, such as viability at 25°C, longevity reduced at this temperature only to about 50%, delayed temperature-sensitive paralysis and fast recovery (Eberle, 1995). These observations support the above proposal, that the strong phenotype shown by the cysteine string mutants is not mainly due to incomplete or incorrect targeting of the mutated protein but that the cysteine string is important for CSP function beyond its demonstrated role in targeting of the protein to the synaptic vesicle membrane. This hypothesis can possibly be tested by replacing the cysteine string with other synaptic vesicle membrane-targeting signals.

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