# Cysteine String Proteins: A Potential Link Between Synaptic Vesicles and Presynaptic $Ca^{2+}$ Channels

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**Presynaptic calcium channels are key regulators of neurotransmitter release. Oocyte expression studies suggest that cysteine string proteins are essential subunits or modulators of these channels. Subcellular fractionation revealed that cysteine string proteins copurify with synaptic vesicles. An average vesicle had eight protein monomers with both the amino and carboxyl termini detected on the cytoplasmic face. Thus, docked synaptic vesicles may regulate presynaptic calcium channels and neurotransmitter release.**

Secretion of neurotransmitter is triggered by calcium ions that permeate the nerve terminal through voltage-gated calcium channels (*1*, *2*). Considerable progress has been made to identify calcium channel subtypes that control this release process (*2*, *3*). Recent efforts (*4*) to clone complementary DNAs (cDNAs) encoding subunits of vertebrate presynaptic calcium channels culminated in the identification of a *Torpedo* cyteine string protein (Tcsp) (*5*, *6*). Electrophysiological data indicated that this protein was an essential subunit or modulator of presynaptic calcium channels (*4*). On this basis, Tcsp was expected to be a plasma membrane protein. Instead, we report that Tcsp copurifies with synaptic vesicles.

We used *Torpedo* electric organ to obtain highly purified synaptic vesicles (*7*, *8*). The final stage in the purification of these vesicles was a chromatographic step that resolved the synaptic vesicles (peak 2 of Fig. 1A) from heterogeneous membrane fragments (peak 1 of Fig. 1A) (*7*–*9*). The presence of occluded acetylcholine (Fig. 1A) and the $SV_2$ antigen (Fig. 1B), an established vesicular protein (*10*), confirmed that peak 2 contains synaptic vesicles. Immunoreactivity of Tcsp was absent in peak 1 (Fig. 1) and was detected only in fractions that contained synaptic vesicles (Fig. 1C), thus indicating that Tcsp associates specifically with these organelles.

Using the immunoblot procedure of Fig. 1, we were able to detect a band (about 1 ng) of Tcsp immunoreactivity from 100 ng of synaptic vesicle protein (*11*, *12*). Thus, Tcsp is 1% of synaptic vesicle protein. Because a single synaptic vesicle has $20 \times 10^3$ kD of protein (*13*), Tcsp accounts for 200 kD, and the average vesicle will have eight Tcsp monomers (*11*).

*Torpedo* cysteine string protein behaves as an intrinsic membrane protein, as evidenced by the requirement for detergent to solubilize it (*9*). To establish the membrane topology of Tcsp, we used antibodies specific for its $NH_2$- or COOH-termini (*14*). Both of these antibody preparations selectively immunoprecipitate Tcsp and $SV_2$ immunoreactivity, occluded acetylcholine, and morphologically identifiable synaptic vesicles (Fig. 2) (*14*, *15*). Because Tcsp is fatty acylated on multiple cysteine residues (*12*), we postulate that it is tethered by these acyl residues to the cytoplasmic surface of vesicles with the $NH_2$- and COOH-termini accessible from the cytoplasm.

As a synaptic vesicle protein, how might Tcsp regulate presynaptic calcium channels? We propose that subsequent to the docking of a vesicle at the plasma membrane, a process involving multiple recognition proteins (*16*), Tcsp interacts with a presynaptic calcium channel. This association converts the channel from a state where it will not open in response to membrane depolarization to a new state where it can open. This interaction may be direct, or it may involve other proteins (*17*, *18*).

Several corollaries emerge from this model. First, docked vesicles should be in close proximity (≤30 nm) to presynaptic

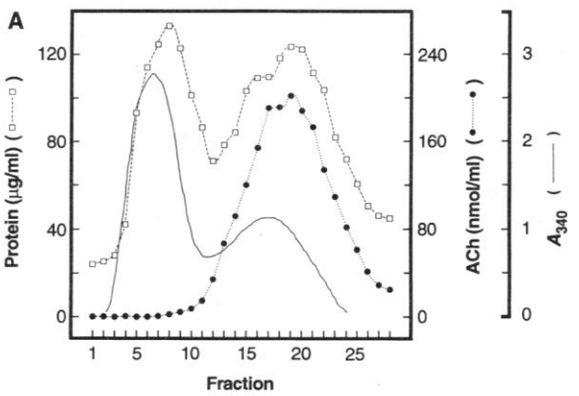


**Fig. 1.** (**A**) Measurements were made of light scattering [absorbance at 340 nm ($A_{340}$)], protein, and acetylcholine (ACh) content of *Torpedo* electric organ membrane fractions resolved by gel filtration. (**B**) Immunoblot assay of $SV_2$ immunoreactivity (some of which failed to enter the resolving gel) in sequential chromatographic fractions. (**C**) Immunoblot detection of Tcsp immunoreactivity in the chromatographic fractions. The immunoreactive bands below the 34-kD species are deacylated variants of Tcsp (*12*). Molecular sizes are indicated on the left in kilodaltons.

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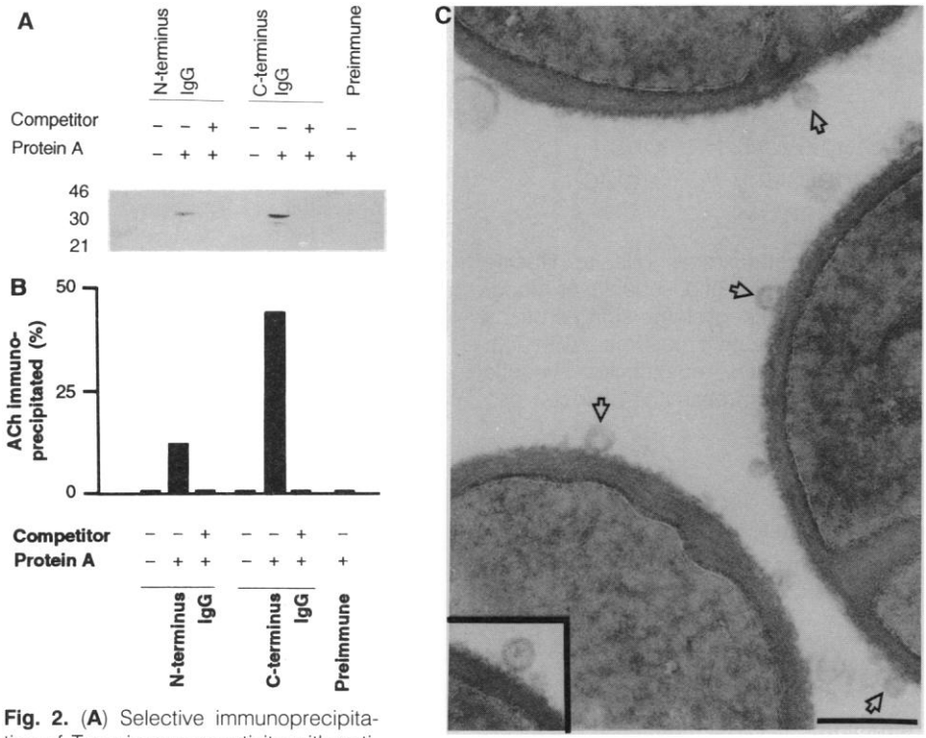


**Fig. 2.** (**A**) Selective immunoprecipitation of Tcsp immunoreactivity with antibodies specific for $NH_2$- or COOH-termini (N- and C-terminus, respectively) of Tcsp. Molecular sizes are indicated on the left in kilodaltons. (**B**) Selective immunoprecipitation of occluded acetylcholine (ACh) with antibodies to Tcsp specific for $NH_2$- or COOH-terminus. (**C**) Electron micrographs of synaptic vesicles bound to *Staphylococcus aureus* immunoadsorbent by COOH-terminal–specific antibodies (arrowheads). Scale bar, 0.33 μm. Inset, adjacent field, same scale.

calcium channels (*19*). Second, presynaptic calcium channels should be capable of opening only after a synaptic vesicle has docked and a csp has interacted with the channel. This constrains calcium ion permeation to sites where vesicles are poised for exocytosis. Third, only a small proportion of csps should be associated with a presynaptic calcium channel at any one time, and conversely, not all presynaptic calcium channels should be in the csp-activated mode. Finally, changes in the number, distribution, or biochemical properties of csps could dramatically affect the triggering of the secretory event. Thus, csps are plausible targets for modulation leading to long-term potentiation or depression of synaptic transmission.

## REFERENCES AND NOTES


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5. This revised nomenclature recognizes the homology of the fish $CCCS_1$ protein (*4*), and *Drosophila* csps (*6*).
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8. Synaptic vesicles were purified as described (*7*) and light scattering (relative apparent absorbance) at 340 nm was used to resolve two peaks of eluting material. Protein was assayed with a kit from Bio-Rad with human immunoglobulin G (IgG) as a standard and acetylcholine was quantitated with a gas chromatographic–mass spectrometric assay [D. J. Jenden, M. Roch, R. Booth, *Anal. Biochem.* **55**, 438 (1973)]. Immunoblot analyses were done as described (*9*) with either monoclonal antibodies against $SV_2$ (*10*) or antibodies against the COOH-terminal of Tcsp (*9*).
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11. The detection limit of the immunoblot procedure and of vesicular Tcsp was estimated by serial dilution of fusion protein or vesicle protein, respectively. The uncertainty of these estimates is less than a factor of two. We used 25 kD as the mass of Tcsp on the basis of its deduced primary sequence (*4*) and extensive fatty acylation (*12*). Cumulative error in the estimate of Tcsps per vesicle also reflects error in the protein per vesicle (*13*) and gives a range of 2 to 20 Tcsp monomers per vesicle.
12. Our unpublished data indicate that native Tcsp has at least 10 fatty acyl groups esterified to cysteine residues [see C. B. Gundersen, A. Mastrogiacomo, J. A. Umbach, *Neurosci. Abst.* **19**, 1329 (1993)].
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14. The protein coding sequence of Tcsp was inserted into the Sal I–Eco RI sites of the pMal vector (NEB Laboratories, Beverly, MA), and antibodies against fusion protein were raised and characterized (*9*). We purified $NH_2$-terminal selective IgG by cleaving the fusion protein with 2-nitro-5-thiocyanobenzoic acid and immobilizing the truncated protein on nitrocellulose for isolation of these IgGs [E. Harlow and D. Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), p. 498]. The COOH-terminal IgGs were those described previously (*9*). Immunoprecipitation of pooled, purified synaptic vesicles (1.5 μg of protein) was in 100 μl of 0.8 M sucrose, 20 mM tris (pH 7.4), 1 mM EGTA, 0.02% sodium azide with 1 to 2 μg of IgG at 4°C. Control samples contained 10 μM COOH-terminal peptide of Tcsp or 2 μg of the fusion protein, respectively. After 4 hours, protein A immunoadsorbent (or, for the controls without protein A, protein A–deficient adsorbent from Sigma) was added for 1 hour, collected by centrifugation, washed with the binding solution, and either processed for acetylcholine content (*8*), electron microscopy [G. A. Zampighi, J. E. Hall, G. R. Ehring, S. A. Simon, *J. Cell Biol.* **108**, 2255 (1989)], or immunoblot of Tcsp or $SV_2$ (*9, 10*). Results for $SV_2$ paralleled Tcsp.
15. Specificity of immunoprecipitation is shown by the absence of detectable immunoprecipitation of Tcsp or occluded acetylcholine using preimmune IgG, protein A–deficient immunoadsorbent, or competitors (peptide or fusion protein).
16. T. Sollner *et al.*, *Nature* **362**, 318 (1993); T. Sudhof and R. Jahn, *Neuron* **6**, 665 (1991); W. S. Trimble, M. Linial, R. H. Scheller, *Annu. Rev. Neurosci.* **14**, 93 (1991).
17. There is precedent for subunit modulation of the biophysical properties of calcium channel $\alpha_1$ subunits (*18*) and for independent G protein control [R. J. Miller, *FASEB J.* **4**, 3291 (1990); B. Hille, *Neuron* **9**, 187 (1992)]. Also, Tcsp harbors a J domain [A. J. Caplan, D. M. Cyr, M. G. Douglas, *Mol. Biol. Cell* **4**, 555 (1993)] that can interact with Hsp70 proteins, which copurify with other components of the vesicle-docking complex (*16*). An explanation of the original electrophysiological results for csp modulation of expressed calcium channels (*4*) is that these protein-protein interactions must have been reconstituted in the oocyte. Whether this was achieved by soluble Tcsp or Tcsp bound to constitutive or cortical secretory organelles remains to be determined.
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19. Recent biochemical [for example, V. M. O'Connor, O. Shamotienko, E. Grishin, H. Betz, *FEBS Lett.* **326**, 255 (1993)] and physiological data [E. Stanley, *Neuron* 11, 1007 (1993)] support this conclusion. The upper limit (30 nm) derives from secondary structure predictions for the $NH_2$-terminus of Tcsp.


20. We thank H. Johnson for preparation of synaptic vesicles, M. Roch and K. Rice for help with acetylcholine assays, M. Kreman for assistance with electron microscopy, and R. Edwards for $SV_2$ antibodies. Supported by NIH and a gift from Mr. and Mrs. M. Libow.

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