Cysteine string proteins (Csps) are synaptic vesicle proteins thought to be involved in calcium-dependent neurotransmitter release at nerve endings. Here, we report the cloning of two Csp variants, termed Csp1 and Csp2, from bovine adrenal medullary chromaffin cells. The bovine Csp1 appears to be the homologue of rat brain Csp, sharing 95% identity at the amino acid level. The nucleotide sequence of csp2 is identical with that of csp1 except for a 72-base insert which introduces a stop codon into the coding sequence, which would be predicted to result in a truncated protein 3.3 kDa smaller than Csp1. Furthermore, polymerase chain reaction analysis detected homologues of Csp1 and Csp2 in rat kidney, liver, pancreas, spleen, lung, and adrenal gland. Expression of Csps in non-neuronal tissues was confirmed by Northern blotting and by immunoblotting with anti-Csp1 antiserum which also demonstrated expression of both full-length and truncated Csps in spleen. The widespread tissue distribution is inconsistent with a role for Csps as specific regulators of presynaptic calcium channels as previously proposed. We suggest that Csps may have a more general role in membrane traffic in non-neuronal as well as neuronal cells.

Calcium influx stimulates the secretion of neurotransmitters and catecholamines from presynaptic nerve terminals and adrenal chromaffin cells, respectively (1). Although calcium is the trigger for regulated secretion, the underlying mechanisms of exocytosis are controlled at the level of protein-protein interactions. Identification and characterization of proteins involved in secretion has recently become the focus of intensive research (2, 3), and adrenal chromaffin cells have been an important model for the study of neuroendocrine secretion (2).

Cysteine-string proteins (Csps) were originally discovered in Drosophila, where they were apparently found localized exclusively at synaptic terminals (4). Csps are unique in that they contain a cysteine-rich motif, which in Drosophila consists of 11 cysteine residues flanked on either side by another pair of cysteines. The proteins also incorporate a "J" domain, homologous to DnaJ proteins which interact with the bacterial homologue of the chaperone protein Hsp70 (5). Drosophila Csps exist in at least two forms generated by alternative RNA splicing (4), and a single related Csp was found in Torpedo (6).

An independent study found that Torpedo csp antisense cRNA virtually abolished expression of an N-type calcium channel in Xenopus oocytes injected with Torpedo mRNA (6). Torpedo Csp was, therefore, proposed to be an essential subunit or modulator of presynaptic calcium channels. However, Csps were subsequently found to copurify with synaptic vesicles and not with presynaptic membranes (7), prompting the proposal that Csps on the synaptic vesicle membrane may interact with presynaptic calcium channels converting them from an inactive to an active state. Torpedo Csp has been shown to be post-translationally modified by the palmitylation of 11 or 12 of the 13 cysteine residues, and this fatty acylation is thought to tether Csps to the synaptic vesicle membrane (8).

Genetic analysis found that deletion of the entire Drosophila csp locus or the promoter sequence and first exon conveyed a temperature-sensitive lethal phenotype which was subsequently characterized as causing a defect in presynaptic neurotransmission (9, 10). The fact that the mutant phenotype was more pronounced at 30 °C than at 22 °C suggests that Csps may stabilize components of the neurotransmitter release machinery. Interestingly, deletion of dnaJ in Escherichia coli causes a temperature-sensitive phenotype suggestive of chaperone activity (11).

A Csp has recently been cloned from rat brain, suggesting that these proteins may also play an important role in mammalian presynaptic neurotransmission (12). It has been suggested that Csps function in membrane fusion (13), but their exact role in the nerve terminal is still not known. In this paper we report the cloning of two csp coding sequences from bovine adrenal medullary chromaffin cells. The first encodes a protein identical in size with the rat brain form, whereas the other, which appears to be a splice variant of the first, encodes a truncated protein approximately 3.3 kDa smaller in size. Subsequent PCR, Northern, and immunoblotting analysis revealed that Csps are not brain-specific as previously reported (4), but are in fact found in a range of non-neuronal tissues, suggesting a more general function for these proteins.

**Experimental Procedures**

Materials—Restriction enzymes, Taq polymerase, T4 DNA ligase, reverse transcription system, and plasmid purification kits were all obtained from Promega. RNeasy RNA isolation kit, pQE-30 plasmid DNA, Ni2+–NTA agarose, and E. coli M15[pREP4] cells were obtained from Qiagen (Surrey, UK). DNA primers for PCR reactions were obtained from Cruachem (Glasgow, UK). Multiple tissue poly(A) RNA blot was obtained from Clontech. 32P-labeled dUTP was obtained from Amersham (Buckinghamshire, UK). All other chemicals were of analytical grade from Sigma Ltd. (Poole, UK).

PCR Amplification of csp Coding Sequences—RNA was extracted from freshly isolated adrenal chromaffin cells purified by differential...
plating (14), PC12 cells or the appropriate rat tissues using a RNase
total RNA isolation kit, following the suppliers protocol (Qiagen). DNA
was synthesized with a Promega reverse transcription protocol, follow-
ing the suppliers protocol. A HYBAID Omnis-E programmable dryblood
was used for PCR reactions. The 5′ primer was designed to contain a 22-
base nucleotide homology to the template, whereas the 3′ primer had
a 24-base nucleotide homology. The primers had BamHI and HindII
restriction sites incorporated to facilitate cloning. The sense and anti-
sense primers used were (5′-dAAGGATCCATGGCTGACCAGAG-
GCAGCG) and (3′-dCTCAAGCTTTTAGTGACCGCTCGGTG), re-
spectively, and the BamHI and HindII restriction sites are underlined.
PCR consisted of an initial denaturation cycle at 95°C for 5 min,
followed by 30 cycles consisting of annealing at 52°C for 1 min, elon-
gation at 72°C for 2 min, and denaturation at 95°C for 1 min. A further
PCR cycle was performed for 10 min after the 30 cycles finished the amplification process. PCR prod-
ucts were examined by electrophoresis on 1.5% agarose gels.

Sequence of csp Coding Regions—The PCR products were gel-
purified using a Promega PCR kit, digested with BamHI and HindII
and ligated to pQE-30 (Qiagen) and the ligation mixture used to trans-
form competent E. coli M15[pREP4] cells (Qiagen). pQE-30 plasmid
DNA was prepared from transformed M15 cells using Wizard plasmid
preps (Promega), and the cloned DNA was sequenced using an auto-
mated sequencer, with sequencing of both strands being carried out.

Expression and Purification of Recombinant Csp1—l-seryl-1-thio-
β-p-galactopyranoside (1–2 mM) was added to a 1-liter culture of M15
cells containing the cloned csp1 coding sequence and incubated at 37 °C
for 5 h with shaking at 250 rpm, inducing expression of the recombinant
protein. The induced cells were pelleted by centrifugation at 4,000 × g
for 20 min and resuspended in 20 ml of breating buffer (5 mM ATP; 100
mM HEPES, 5 mM MgCl2, 2 mM β-mercaptoethanol, 500 mM KCl, 1 mM
phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 1 μM pepstatin
A, pH 7.0). The cells were stored at −20°C overnight and thawed the
following morning. 1 mg/ml lysozyme was added, and the cells were left
on ice for 30 min. Following ultrasonication, 2 μg/ml DNase was added,
and the cells were left on ice for 15 min. Centrifugation at 100,000 × g
for 1 h removed cell debris. The recovered protein fraction was loaded
onto an L-100 agarose column and washed with 50 mM imidazole
buffer (50 mM imidazole, 20 mM HEPES, 200 mM KCl, 2 mM β-mercap-
toethanol, 0.5 mM ATP, 10% v/v glycerol, pH 7.0) to remove unbound
protein. His6-tagged Csp1 was eluted by application of a gradient of
50–500 mM imidazole, and peak fractions containing the recombinant
protein were identified by SDS-polyacrylamide gel electrophoresis. The
procedure yielded ~16 mg of essentially pure recombinant Csp1 at a
concentration of 1.65 mg/ml. Chromatography was performed at 4°C
using a Pharmaic FPLC system.

Northern Blotting—A multiple tissue poly(A+) RNA blot (Clontech)
was used for Northern blotting. Prehybridization and hybridization
were performed at 65 °C in 5 × SSPE containing 50% deionized form-
amide, 5 × Denhardt’s, 0.5% SDS, and 0.2 mg/ml salmon sperm in
diethyl pyrocarbonate-treated water. A complementary RNA probe to Csp
coding sequence but incorporating restriction sites to allow
amplification from cDNA. The DNA species were amplified by PCR from cDNA
using primers designed to the 5′ and 3′ ends of the rat brain csp coding
sequence with restriction sites incorporated. Amplification from chro-
maffin cell and PC12 cell cDNA generated two DNA species (indicated by arrows), the smaller of these being identical in size with that
generated from rat brain cDNA.

RESULTS

Since CspS are highly conserved, a strategy was adopted to allow the PCR
amplification of the coding sequence of bovine chromaffin Csp using oligonucleotide primers based on the rat
brain csp sequence but incorporating restriction sites to allow
direct cloning into vectors for expression of His6-tagged proteins.
In initial experiments, mRNA was isolated from purified bovine adrenal medullary chromaffin cells, the rat clonal PC12
pheochromocytoma cell line, and rat brain. Reverse transcrip-
tion of the mRNA was used to generate complementary cDNA
which was subsequently amplified by PCR using oligonucleo-
tide primers designed to the 5′ and 3′ regions of the rat csp
coding sequence. The result of the PCR amplifications is shown
as Fig. 1. Only one size of DNA fragment was amplified from
rat brain, and this is in agreement with a previous study (12),
suggesting the presence of only a single csp species in rat brain.
However, a slightly larger DNA molecule was amplified from
chromaffin and PC12 cell cDNA in addition to a fragment
apparently identical in size with that from rat brain.

The DNA fragments obtained from PCR amplification of chromaffin cell cDNA were purified, ligated to BamHI/HindII-
digested pQE-30, and transformed into E. coli M15[pREP4]
cells. Of 9 clones that were isolated, 3 contained a smaller PCR
product. From sequencing, 2 encoded the likely bovine homo-
logue of the rat brain csp (Fig. 2). This species which we term
bovine Csp1 showed 88% nucleotide and 95% amino acid se-
quence identity with the rat brain Csp. Six clones encoded a likely splice variant, which we term Csp2, since it was identical
with the first coding sequence apart from a 72-base insert
(GGAGGGCACTGACCTGTCGCGGAGTGGTTTGCGTGGGCA-

**FIG. 1. Amplification of rat brain, chromaffin cell, and PC12 cell csp DNA.** The DNA species were amplified by PCR from cDNA
using primers designed to the 5′ and 3′ ends of the rat brain csp coding
sequence with restriction sites incorporated. Amplification from chro-
maffin cell and PC12 cell cDNA generated two DNA species (indicated by arrows), the smaller of these being identical in size with that
generated from rat brain cDNA.

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**FIG. 2.** Tissue homogenates were prepared from Wistar rats by homogenization in 5 mM Tris-HCl (pH 8.0), and SDS-dissocia-
tion buffer was added to give a final protein concentration of 1 mg/ml.
2 mM dithiothreitol was added, and the homogenates were left at room
temperature for 1 h before boiling for 5 min. The boiled samples were
separated on a 12.5% polyacrylamide gel. Transfer of proteins to nitro-
cellulose was then incubated with antiserum raised in

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**FIG. 3.** Membrane immunoblotting of crude membranes from PC12 cells. 20 μg of total protein was electrophoresed and
blotted on nitrocellulose. After blocking, the membrane was incubated
with antiserum raised in

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**FIG. 4.** SDS-PAGE of purified recombinant Csp1. 15 μg of protein was resolved on a 12.5% polyacrylamide gel, and the
bands were stained with Coomassie blue.

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**FIG. 5.** Immunofluorescence staining of PC12 cells. The cells were cultured on glass coverslips and fixed with 4% formaldehyde
for 10 min, permeabilized with 0.1% saponin, and incubated with antiserum raised in

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**FIG. 6.** Immunoblotting of cell extracts with antiserum raised in

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**FIG. 7.** Immunoprecipitation of recombinant Csp1 from PC12 cell lysates. The cleared lysate was incubated with

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**FIG. 8.** Western blotting of recombinant Csp1. 15 μg of protein was resolved on a 12.5% polyacrylamide gel, and the
bands were stained with Coomassie blue.
Ni
dalyzed (data not shown). The induced proteins binding to the D-galactopyranoside to transformed bacterial cultures. The ex-
tional insert present in the coding sequence of rat brain csps, csp DNA was amplified from all tissues examined but with apparent differential expression of the two csps in the different tissues. b, RNA blots containing poly(A)-enriched RNA were hybridized with a probe encompassing the coding region of Csp1 and the hybridization signal was detected using a PhosphorImager.

During the PCR procedure, all clones were ampliﬁed to eliminate problems due to PCR amplification errors. In addition, all nucleotide differences between bovine, rat, and Drosophila Csps can express two distinct Csps. DNA sequencing demonstrated that the two plasmid inserts encode variant Csps, possessing this insert but also had an 88-base deletion at positions 492 and 234 which would result in a reading frameshift after amino acid 78. This last clone was not characterized further. All tissues examined resulted in the amplification of two cDNA bands identical in size with DNA-encoding Csp1 and Csp2, but with differing ratios of the two products from the various tissues. The presence of mRNA-encoding Csps was conﬁrmed by Northern blotting. RNA blots loaded with poly(A)-enriched RNA from various rat tissues were hybridized at high stringency with an RNA probe complementary to the Csp1 coding region, and mRNAs were detected in heart, spleen, lung, liver, muscle, kidney, and testis in addition to brain (Fig. 3b). In each tissue, an mRNA species of around 5 kilobases was detected which was more abundant in brain and brain also contained a larger mRNA.

To directly conﬁrm expression of Csp in non-neuronal tissues, an antiserum was raised using puriﬁed expressed Csp1 as the antigen. The antiserum recognized both Csp1 and Csp2 recombinant proteins, and, in immunoblotting on rat brain homogenates, recognized a polypeptide of around 36 kDa as expected for the post-translationally modiﬁed Csp1 (Fig. 4). The antiserum also speciﬁcally recognized a larger polypeptide of around 70 kDa, the amount of which varied between experiments. It is well established that Csps in Torpedo and rat brain form dimers detectable by immunoblotting (12, 16), and it is likely that the antiserum recognizes only Csp1 and its dimer in brain homogenates. The antiserum also recognized an abundant 36-kDa polypeptide and the dimer in chromaffin cells. Since chromaffin cells are derived from neuronal precursor cells, we examined the possible non-neuronal expression of Csp in kidney and spleen, tissues that would not be expected to contain any neuronal-like cells. Immunoblotting showed less abundant but detectable expression of Csp1 in both kidney and spleen. Polypeptides corresponding to Csp dimers were also seen in these tissues. Immunoreactivity was also detected in liver and pancreas, but this was at the limit of detectability. An immunoreactive polypeptide, corresponding in size to that expected for post-translationally modiﬁed Csp2, was detected in spleen (Fig. 4).

**DISCUSSION**

We have cloned and characterized two Csp variants from bovine adrenal chromaffin cells. PCR ampliﬁcation of cDNA from the clonal PC12 cell line also generated two DNA species identical in size with csp1 and csp2 conﬁrming that one cell type can express two distinct Csps. DNA sequencing demonstrated that the two plasmid inserts encode variant Csps,
which are highly homologous at the nucleotide and amino acid level to the previously identified Csp1s. Protein expression confirmed that proteins of the correct molecular mass were synthesized and most notably that csp2 encodes a truncated protein, as predicted by DNA sequence analysis. The truncated Csp2 has intact "J" and cysteine-rich domains and is thus a Csp, but lacks the extreme C terminus possessed by the other members of the Csp family. Future studies of the functions of Csp1 and Csp2 should indicate the role of the C-terminal domain.

Previous studies have only detected one Csp protein isoform in both rat brain and Torpedo electric lobe (12, 15). However, in both cases, detection was by Western blotting using antibodies specific for the C-terminal region of Torpedo Csp. These antibodies would clearly not bind to the truncated Csp2 which lacks the C terminus. The present study failed to identify by PCR a larger DNA species (similar to that encoding Csp2) in rat brain, and it may well be that brain expresses only the full-length Csp1.

Using PCR we have detected expression of the two csp mRNAs in rat kidney, liver, spleen, pancreas, lung, and adrenal gland (all tissues examined), and Northern blotting detected mRNAs in brain, heart, spleen, lung, liver, muscle, kidney, and testis, implying that Csps are widespread. This finding is in contrast to previous work which has claimed that Drosophila Csps are synapse-specific (4), and that csp mRNA is not present in electric organ, liver, or muscle of Torpedo (15, 16). Analysis of protein expression by immunoblotting with antiserum raised against recombinant Csp1 confirmed expression in chromaffin cells and other non-neuronal tissues. Csp2 was readily detected only in spleen suggesting that this variant protein is expressed at low levels. In immunoblotting, the polypeptide corresponding to Csp2 was detected in spleen but not in kidney despite similar amounts of the two PCR products being detected in both these and other tissues. It should be noted, however, that the PCR method used was not quantitative and the relative amounts of the two PCR products would not reflect the relative amounts of the mRNAs for the two Csps in these tissues. The ratio of the Csp dimer to monomer detected by immunoblotting varied considerably between different tissues. The significance of these differences is not clear since the ratio of dimer to monomer varied widely between experiments even with the recombinant Csp1. We were unable to find conditions that prevented dimer formation. While this paper was under review, expression of Csp1 was demonstrated in adrenal medulla (17) consistent with our findings. Interestingly, a number of proteins involved in exocytosis previously thought to be brain-specific have since been found in non-neuronal cell types, most recently the Ca\(^{2+}\)-sensing protein synaptotagmin (18), setting a precedent for the present finding.

The implications of the apparently ubiquitous tissue distribution of csp are manyfold. It has been suggested that Csps may regulate voltage-dependent Ca\(^{2+}\) channels (6) and mediate membrane fusion (13). Our finding that Csps are not restricted to synapses makes their prime function unlikely to be the regulation of Ca\(^{2+}\) channels as many of the tissues examined do not have such channels. Alternatively, Csps may be universal membrane traffic proteins such as NSF and SNAP50, which function in both constitutive and regulated secretion. It is possible that Csps could mediate membrane fusion as suggested previously (13), but our findings do not restrict the proteins to the synapse. If Csps do function in membrane fusion at the synapse during neurotransmission (13), then it seems possible that they also play a fundamental and more general role in membrane traffic within the cell.

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Note Added in Proof—The bovine Csp1 sequence shows greater identity, with only four amino acid differences, to a more recently reported rat brain Csp sequence, and non-neuronal expression of Csp was shown in pancreatic zymogen granules (13).

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Identification of a Novel Cysteine String Protein Variant and Expression of Cysteine String Proteins in Non-neuronal Cells
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