FEBS 17357 FEBS Letters 391 (1996) 269–272

Widespread expression of human cysteine string proteins

Thierry Coppola, Cameron Gundersen*

The Department of Molecular and Medical Pharmacology, UCLA School of Medicine, Los Angeles, CA 90095, USA

Received 17 May 1996; revised version received 21 June 1996

Abstract We present the nucleotide and deduced amino acid sequences of a human cysteine string protein (csp) and a unique truncated csp variant that derives from the retention of an exonic sequence that introduces a premature, in-frame stop codon. Low stringency Southern analysis is compatible with the presence of a single human csp gene. Northern analysis reveals that human csp mRNA has both a more heterogeneous size distribution and a more widespread tissue distribution than previously reported for other csps. Exemplifying this is the fact that csp immunoreactivity is detected in Triton X-114 extracts of human blood, an observation which may facilitate blood-based diagnostic assays of csp status in man.

Key words: Cysteine string protein; Secretory vesicle; cDNA cloning; Northern analysis

1. Introduction

Cysteine string proteins (csps) are extensively lipidated, cysteine-rich proteins that are prominently localized at nerve endings of vertebrates and invertebrates [1–4]. More recently, csps have also been detected in exocrine and endocrine secretory cells [4,5]. Csps are important for the normal release of neurotransmitter at skeletal neuromuscular junctions in *Drosophila* [6], and they have been implicated both in the regulation of presynaptic calcium channels and in other phases of the secretory cascade [7–9]. Because of the neurological deficits and premature death of *Drosophila* csp mutants [6,9] and the possibility that defective csps may underlie disturbances of secretory and nervous system function in man, we cloned and sequenced human csp cDNAs and undertook a preliminary characterization of human csps. Several interesting observations emerged: (i) we obtained a human csp cDNA that predicts a truncated form of csp that is shortened by about 30 amino acid residues; (ii) low stringency Southern analysis implies that there is only a single human csp gene; (iii) relative to other species, Northern analysis reveals appreciably more heterogeneity in the mass distribution of human csp mRNAs as well as in the tissues where these mRNAs are detected; (iv) finally, we provide immunoblot evidence that csps are present in human blood, an observation that may have practical relevance for screening for structural disturbances of csps in man.

2. Materials and methods

2.1. Cloning and sequencing human csp cDNA

A partial human csp cDNA clone was isolated by screening 6×10^5 clones from a human brain lambda Zap II cDNA expression library (Stratagene) using affinity-purified antibodies against a recombinant rat csp that had the cysteine string deleted (pilot experiments showed that these antibodies recognize a csp-like protein on immunoblots of membrane protein from post-mortem human brain). The nucleotide sequence of the 0.75 kb insert of this clone revealed that it was highly homologous to other csp cDNAs in that it encoded a J-domain and a cysteine string (e.g. see [2]). However, this initial clone lacked the initiation codon, so we used it as a probe to isolate additional cDNAs. Perhaps owing to secondary-structure complications, none of these additional clones (with inserts ranging from 1.2 to 3.0 kb) contained the 5′ extremity of the csp open reading frame. In the end, we isolated two independent genomic csp clones (using a human genomic DNA library in lambda Dash II from Stratagene) with approximately 12 kb inserts. Restriction mapping and nucleotide sequencing of portions of this genomic DNA yielded the missing csp open reading frame and also gave insight into the origin of the truncated form of csp cDNA presented in Section 3. The nucleotide sequence was obtained for both DNA strands using a combination of subcloning and sequence-specific primers.

2.2. Southern analysis of human genomic DNA

Samples of 2 μg of human genomic DNA (Promega) were cut with restriction endonucleases and prepared for Southern analysis exactly as described in [10]. Prehybridization was at 42°C in a solution of 25% formamide, 5×SSPE (1×SSPE = 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA), 5×Denhardt's solution (1×Denhardt's solution is: 1% Ficoll, 1% polyvinylpyrrolidine, 1% bovine serum albumin), 0.5% SDS, and 0.1 mg ml^{-1} of denatured salmon sperm DNA. After 4 h the prehybridization solution was replaced with hybridization solution that was supplemented with dextran sulfate (final concentration: 10%, w:v) and ^{32}P-labeled probe obtained using random-prime labeling of a 1.8 kb, gel-purified, human csp cDNA (that lacks about 30 nucleotides of the 5′ open reading frame). Hybridization was for 20 h at 42°C after which the filter was washed in a flow apparatus (Millipore) with a final low stringency wash using 0.1×SSPE, 0.1% SDS at 50°C. Bands were detected by autoradiography.

2.3. Northern analysis

We purchased a nitrocellulose filter from Clontech that has mRNA from several human tissues. Prehybridization and hybridization conditions were identical to those described for Southern analysis except that high stringency condition of hybridization (with 50% formamide) and wash (at 67°C rather than 50°C) were used, and the probe was the original 0.75 kb human csp cDNA fragment.

2.4. Csps in human blood

Fatty acylated csps partition effectively into the Triton X-114 phase during Triton X-114 phase partitioning experiments [11]. We exploited this fact to develop a procedure to extract csps from human blood samples: normally, 0.5 ml of fresh or frozen whole blood was diluted with 4.5 ml of 10 mM Tris, 1 mM EDTA (pH 7.4 with HCl) and Triton X-114 was added to 1% (v:v). Samples were incubated for 1–2 h at 4°C with gentle agitation and insoluble material was removed by centrifugation for 5 min at 2000×*g*. Samples were layered over a 1 ml cushion (of 6% sucrose in 0.1 M NaCl, 50 mM Tris, 1 mM EDTA with 0.05% Triton X-114 (pH 7.4 with HCl)) in a conical 15 ml centrifuge tube and incubated for 10 min at 37°C. Condensed Triton X-114 was collected by centrifugation at 300–500×*g* for 3 min. Protein was recovered from the Triton X-114 bead using the procedure of Wessel and Flugge [12] and dissolved in SDS sample buffer with 5% SDS. Alternatively samples of the Triton X-114 bead were first treated with a final concentration of 1 M hydroxylamine (pH 7.2–7.5) or 1 M Tris (pH 7.4) with 50 mM dithiothreitol for 2 h at 22°C and then for 2 h with 120 mM iodoacetamide in the dark before the extraction procedure of [12]. Hydroxylamine treatment deacylates csps [3] and iodoacetamide alkylates the exposed thiol groups and reduces the formation of dimeric and multimeric csp complexes [11]. Subsequent

*Corresponding author. Fax: (1) (310) 206-5052.

PII S0014-5793(96)00750-8

immunoblot analysis of csps was as described previously [8,11] using the same affinity-purified anti-csp antibodies that were employed in the cloning experiments. To reduce background signal, the secondary goat anti-rabbit antibodies (conjugated to alkaline phosphatase) were preadsorbed against acetone powder of human blood as described in [13].

3. Results

3.1. Human csp cDNAs and deduced amino acid sequences

In the upper part of Fig. 1, we present the nucleotide and deduced amino acid sequence of human csp cDNA. This form of human csp is designated Hcsp1. The predicted amino acid sequence of Hcsp1 differs only at position 112 (an F for a V) compared to rat csp ([2,5] and see corrected sequence in [14]), and at the nucleotide level there is 88% identity between rat and human csp cDNAs. Thus, these forms of rat and human csp are extremely highly conserved.

Interestingly, one of the eight cDNA clones that we sequenced had a 72 nucleotide insert that introduces an in-frame stop codon in the latter part of the csp open reading frame. This is indicated in the lower part of Fig. 1, where we show the nucleotide and predicted amino acid sequence of this shortened form of human csp (designated Hcsp2). The nucleotide sequence of Hcsp2 cDNA prior to and after the 72 nucleotide insert is identical to Hcsp1 (Fig. 1). However, the carboxy terminus of Hcsp2 is distinctly different from Hcsp1 (Fig. 1). It ends with two glycine residues and a histidine (Fig. 1). This changes appreciably the net charge of Hcsp2 (it is less acidic than Hcsp1), and it also removes a region rich in serine and threonine residues whose function is currently unknown.

Based on sequence analysis of human csp genomic clones, Hcsp2 arises from the retention of a small 72 nucleotide exon that otherwise is removed from the mRNA encoding the Hcsp1 (results not shown). In spite of this truncation, both csp forms retain the characteristic cysteine string and a J-domain [1,2,5,7], the latter motif presumably is involved in interactions with Hsp70 proteins [15].

3.2. Southern analysis

Using as a probe a 1.8 kb human csp cDNA which begins approximately at nucleotide 30 in Fig. 1 (top), low stringency Southern analysis of human genomic DNA reveals a pattern (Fig. 2) that is consistent with the presence of a single human csp gene. For instance, a single band between 4 kb and 5 kb is detected in genomic DNA digested either with *Bam*HI, *Kpn*I or *Hin*dIII. Concomitantly, *Sac*I is known to have single recognition site in the 1.8 kb human csp cDNA probe, and it produces the expected pair of bands. *Hin*cII has multiple recognition sites in the csp gene and produces six distinct bands ranging from about 0.4 kb which is barely visible to 7 kb (Fig. 2). Overall, these results and preliminary analysis of the Hcsp genomic clones reveal that the human csp gene spans in the order of 15–20 kb (Fig. 2).

3.3. Northern analysis

Northern analysis reveals multiple csp mRNA species under high stringency conditions (Fig. 3). Except in kidney where only three bands were evident, all other tissues revealed at least four candidate csp mRNA species of about 1.3 kb, 3.5 kb, 5 kb and 6 kb (Fig. 3). In addition, there was a fifth weak band in human muscle at about 1.8 kb. Interestingly, there is appreciable variation among the different tissues in the relative intensity of the various putative csp mRNAs. For instance, the 1.3 kb species is most prominent in liver while the higher mass mRNAs are less abundant. The converse is true of csp mRNA in the brain and pancreas (Fig. 3).

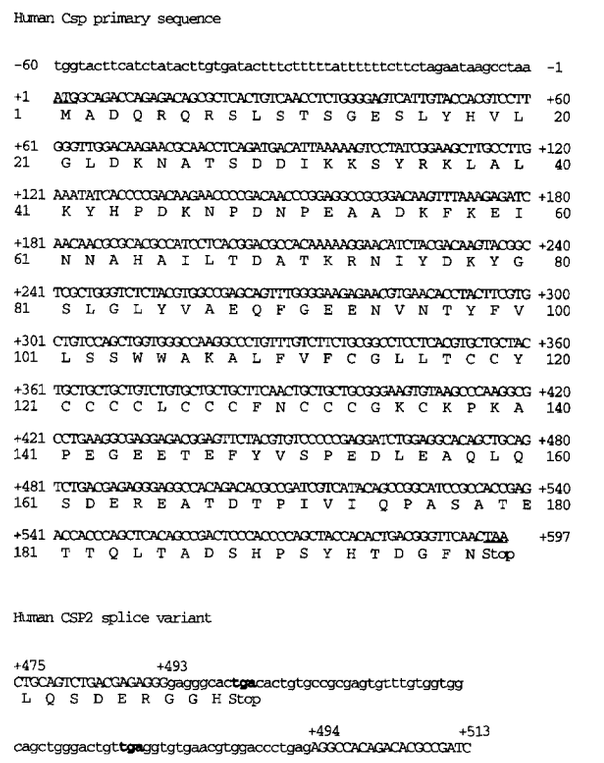

Fig. 1. The nucleotide and deduced amino acid sequences of Hcsp1 and Hcsp2. (Top) The nucleotide and predicted amino acid sequence of Hcsp1 using the single letter convention. (Bottom) Hcsp2 has the same nucleotide sequence as Hcsp1 except for a 72 nucleotide insert shown here. This insert introduces two in-frame stop codons (in bold). The first stop codon predicts a truncated csp variant that is 167 amino acids rather than the 198 residues of Hcsp1.

3.4. Immunoreactive csp in human blood

In pilot experiments, we confirmed that our antibodies against recombinant rat csp recognized csp on immunoblots of human brain membranes (data not shown). Given the high homology between rat and human csps, this antibody cross-reactivity is not surprising. Thus, with the Northern data implying a widespread tissue distribution of csps, we were interested in assessing whether csps might also be detected in human blood. As indicated in Fig. 4 (lane A), a candidate csp immunoreactive band is detected at about 35 kDa. Evidence that this immunoreactive protein is csp comes from the finding that this species displays a 7 kDa downward shift in apparent mass after treatment with the deacylating agent, hydroxylamine (Fig. 4, lane B). Treatment of the Triton X-114 extract of human blood with 1 M Tris (pH 7.4) instead of hydroxylamine does not produce any apparent mass shift (Fig. 4, lane C) which is compatible with the effect of hydroxylamine being due to deacylation of csp. Similar shifts in csp mass are seen for deacylated fish and rat csps [2,3].

4. Discussion

Human csp (Hcsp1) is remarkably similar to rat csp with

only a single predicted change at the amino acid level. This result indicates that evolution of the csp gene is relatively slow, a fact that may reflect the critical importance of csps to neurosecretion, or to secretion in general. Indeed, a similar high degree of primary sequence conservation has been seen in another synaptic vesicle protein, synaptotagmin, which shows 97% identity between rat and man [16]. However, we also obtained evidence for a second form of human csp based on cDNA sequence analysis. Precedents exist in *Drosophila* for csp splice variants [1,9], but it was only very recently that a vertebrate csp splice variant was reported [17]. Interestingly, it appears that the human csp splice variant derives from the same processing mechanism as the bovine form [17]. It will be important to determine whether this truncated form of human csp exhibits a differential cellular (or subcellular) distribution as has been documented for insect and bovine csp splice variants [9,17].

Southern analysis at low stringency suggests that there is a single human csp gene. This conclusion should remain provisional until a more systematic search is made for other genes encoding csp-like proteins. Again, this is exemplified by reference to synaptotagmin. Li and colleagues [18] recently reported four new synaptotagmin isoforms that are distinct variants of the four previously identified forms of this protein. Nucleotide sequences imply that these synaptotagmin isoforms derive from unique genes, so this situation establishes a strong precedent for genetic and primary sequence diversity among proteins associated with secretory organelles. On this basis, it is possible that additional genes encoding csp-like proteins will be found.

An interesting conclusion that emerges from a comparison of the Southern and Northern data is that the multiple mRNAs detected on the Northern almost certainly derive from transcription of the single csp gene detected by Southern analysis. This is because the genomic Southern analysis was conducted at low stringency, whereas Northern analysis was at high stringency. Thus, any mRNAs that were not transcribed from the csp gene revealed in Fig. 2 should not have been detected on the Northern. This implies then that the 3–5 csp mRNA species seen in different tissues are probably due either to multiple transcription initiation sites or to differential mRNA processing. Presumptive evidence for at least one csp

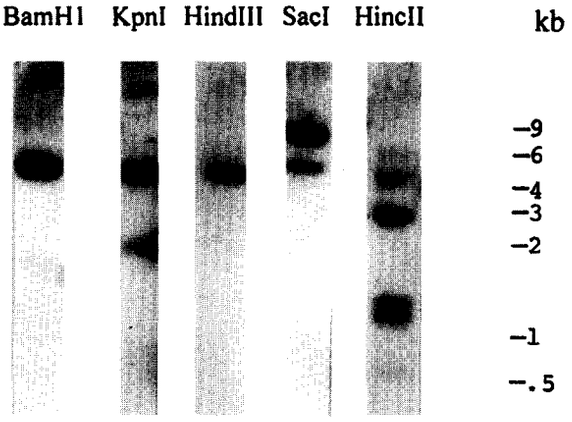

Fig. 2. Human csp genomic Southern analysis. Human genomic DNA was digested with the indicated enzymes and probed at low stringency with a 1.8 kb csp cDNA probe. Bound probe was detected by autoradiography and approximate masses (in kb) are indicated.

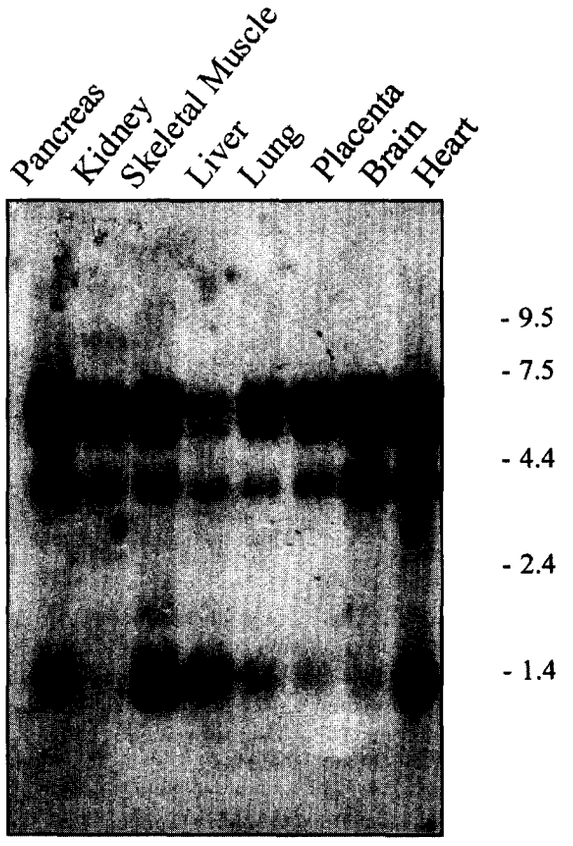

Fig. 3. A human tissue Northern blot was probed at high stringency using a 0.75 kb human csp cDNA probe. Bound probe was detected by autoradiography and mRNA masses are relative to standards (in kb).

splice isoform is seen in the cDNA sequence for Hcsp2. The retention of a 72 nucleotide exon predicts a shortened form of csp with a modified C terminus relative to the original csp isoform. Thus, these two csp isoforms can account for two of the five csp mRNAs detected by Northern analysis, but further work will be needed to correlate specific csp isoforms with each mRNA.

Besides the mass diversity of csp mRNAs, a second striking feature of the Northern analysis is the widespread tissue distribution of csp mRNAs in man. This result was somewhat unexpected because in situ hybridization in *Drosophila* [1] and Northern analysis in *Torpedo* [19] had suggested that csp mRNAs were preferentially localized to nervous tissue. Although more recent work [4,5] has shown that csps are present in exocrine and endocrine secretory cells, the detection of csp mRNA in kidney, liver and placenta ran contrary to expectation. Interestingly, Chamberlain and Burgoyne [17] recently reported evidence from Northern analysis, RT-PCR, and immunoblots that csps are present in a variety of tissues, including, liver, spleen and kidney. These data were interpreted as suggesting that csps play a more generalized role in secretion, rather than a specialized function in calcium channel modulation as previously proposed [8]. At one level, our results are compatible with this interpretation. Thus, we detect csp mRNA in a wide range of human tissues. However, what is needed is a clear indication of which cells in these tissues express csp. This cellular resolution is important for

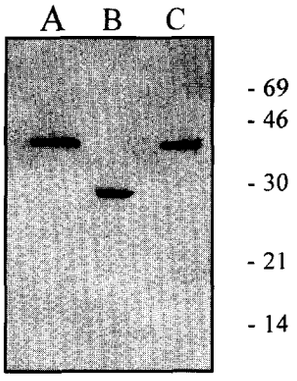

Fig. 4. Immunoblot analysis of human blood samples. Protein recovered from Triton X-114 extracts of human blood (equivalent to 0.15 ml) was subjected to immunoblot analysis using anti-csp antibodies. Lane A: untreated control; lane B: deacylated sample (using hydroxylamine); lane C: sample treated with Tris as a control for hydroxylamine treatment. Note that the csp immunoreactive protein shows about a 7 kDa shift in apparent mass after deacylation in hydroxylamine.

the following reason. Most, if not all, vertebrate neurons express csps [4]. Thus, in any given tissue, intrinsic neurons can contribute to the detectable csp immunoreactivity and csp mRNA. However, even in tissues with few or no intrinsic neurons (e.g. placenta), this issue is not resolved, because csps are present in blood. Therefore, further work will be necessary to validate the conclusion that csps participate more generically in secretory processes.

It is reasonable to enquire what csps might be doing if their cellular distribution is as widespread as our results and those of Chamberlain and Burgoyne [17] suggest (with the above caveats). We previously advanced a model of csp function that is based on the unusual degree of fatty acylation of these proteins [20]. In recognition of the dramatic precedents for the similarity among many protein constituents of the secretory pathway (reviewed in [21–23]), we speculated that csps might participate in events that are critical for membrane fusion, not only in neurons, but in other cells, as well [20]. Thus, these recent results that argue for a broad cellular distribution of csps, may reflect this general role of csps in membrane trafficking.

With the findings that csps are detectable in various non-neuronal secretory cells [4,5], we were interested in determining whether csps could be detected in blood. In particular, because platelets and granulocytes exhibit regulated secretion, we expected that they might express csp-like proteins. Our results support this hypothesis. Using a Triton X-114 extraction protocol, which excludes the vast excess of soluble hemoglobin in whole blood samples, we detected csp immunoreactivity in extracts from modestly sized samples of blood (0.1–0.5 ml). Again, additional work will be necessary to identify the cell(s) that harbor csps, but the presence of these proteins in blood offers the opportunity of using blood samples to assess structural perturbations of csps in man.

Acknowledgements: We thank Dr. A. Mastrogiacomo for the anti-csp antibodies used in this work and Drs. J. Umbach and A. Mastrogiacomo for helpful discussions. T. Sama kindly assisted with preparation of the manuscript. This work was supported in part by a grant from NIH (NS31517).

References

[1] Zinsmaier, K., Hofbauer, A., Heimbeck, G., Pflugfelder, G.O., Buchner, S. and Buchner, E. (1990) J. Neurogenet. 7, 15–29.
[2] Mastrogiacomo, A. and Gundersen, C.B. (1995) Mol. Brain Res. 26, 12–18.
[3] Gundersen, C.B., Mastrogiacomo, A., Faull, K. and Umbach, J.A. (1994) J. Biol. Chem. 269, 19197–19199.
[4] Kohan, S.A., Pescatori, M., Brecha, N.C., Mastrogiacomo, A., Umbach, J.A. and Gundersen, C.B. (1995) J. Neurosci. 15, 6230–6238.
[5] Braun, J.E.A. and Scheller, R.H. (1995) Neuropharmacology 34, 1361–1369.
[6] Umbach, J.A., Zinsmaier, K.E., Eberle, K.K., Buchner, E., Benzer, S. and Gundersen, C.B. (1994) Neuron 13, 899–908.
[7] Gundersen, C.B. and Umbach, J.A. (1992) Neuron 9, 527–537.
[8] Mastrogiacomo, A., Parson, S.M., Zampighi, G.A., Jenden, D.J., Umbach, J.A. and Gundersen, C.B. (1994) Science 263, 981–982.
[9] Zinsmaier, K.E., Eberle, K.K., Buchner, E., Walter, N. and Benzer, S. (1994) Science 263, 977–980.
[10] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, pp. 9.32–9.35. Cold Spring Harbor Press, Cold Spring Harbor, NY.
[11] Mastrogiacomo, A., Evans, C.J. and Gundersen, C.B. (1994) J. Neurochem. 62, 873–880.
[12] Wessel, D. and Flugge, U.I. (1984) Anal. Biochem. 138, 141–143.
[13] Harlow, E. and Lane, C. (1988) Antibodies: A Laboratory Manual, p. 633. Cold Spring Harbor Press, Cold Spring Harbor, NY.
[14] Mastrogiacomo, A. and Gundersen, C.B. (1996) Mol. Brain Res. (in press).
[15] Caplan, A.J., Cyr, D.M. and Douglas, M.G. (1993) Mol. Cell. Biol. 4, 555–563.
[16] Perin, M.S., Johnston, P.A., Ozcelik, T., Jahn, R., Francke, V. and Sudhof, T. (1991) J. Biol. Chem. 266, 615–621.
[17] Chamberlain, L.H. and Burgoyne, R.D. (1996) J. Biol. Chem. 271, 7320–7323.
[18] Li, C., Ullrich, B., Zhang, J.Z., Anderson, R.G.W., Brose, N. and Sudhof, T.C. (1995) Nature 375, 594–599.
[19] Umbach, J.A. and Gundersen, C.B. (1991) Ann. NY Acad. Sci. 635, 443–446.
[20] Gundersen, C.B., Umbach, J.A. and Mastrogiacomo, A. (1995) J. Theor. Biol. 172, 269–277.
[21] Bennett, M.K. and Scheller, R.H. (1993) Proc. Natl. Acad. Sci. USA 90, 2559–2563.
[22] Sudhof, T.C., De Camilli, P., Niemann, H. and Jahn, R. (1993) Cell 75, 1–4.
[23] Burgoyne, R.D. and Morgan, A. (1993) Biochem. J. 293, 305–316.